Long chain fatty acid analogs suppress breast tumorigenesis and progression

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Abstract

Obesity and type 2 diabetes (T2D) are associated with increased breast cancer incidence and mortality, whereas carbohydrate-restricted ketogenic diets ameliorate T2D and suppress breast cancer. These observations suggest an inherent efficacy of nonesterified long-chain fatty acids (LCFA) in suppressing T2D and breast tumorigenesis. In this study, we investigated novel anti-diabetic MEDICA analogs consisting of methyl-substituted LCFA that are neither β-oxidized nor esterified to generate lipids, prompting interest in their potential efficacy as anti-tumor agents in the context of breast cancer. In the MMTV-PyMT oncomouse model of breast cancer, where we confirmed that tumor growth could be suppressed by a carbohydrate-restricted ketogenic diet, MEDICA treatment suppressed tumor growth and lung metastasis, promoting a differentiated phenotype while suppressing mesenchymal markers. In human breast cancer cells, MEDICA treatment attenuated signaling through the STAT3 and c-Src transduction pathways. Mechanistic investigations suggested that MEDICA suppressed c-Src transforming activity by elevating ROS production, resulting in c-Src oxidation and oligomerization. Our findings suggest that MEDICA analogs may offer therapeutic potential in breast cancer and overcome the poor compliance of patients to dietary carbohydrate restriction.
Introduction

Breast cancer is the most common invasive cancer in women. Mammary carcinoma develops in stages consisting of early hyperplastic ductal lesions that progress to adenoma, carcinoma in situ and lung metastasis. Mice expressing the Polyoma Middle T antigen (PyMT) driven by the mammary MMTV promoter (MMTV-PyMT) are widely used as animal model for human breast cancer (reviewed in (1)). Thus, similarly to human tumors, mammary carcinoma in MMTV-PyMT mice develops in stages consisting of early hyperplastic lesions located on end buds of collecting ducts, that progress to adenoma/mammary intraepithelial neoplasia (MIN), carcinoma in situ and lung metastasis (2). PyMT-induced survival is due to a strictly ordered sequence whereby PyMT binds first to the protein phosphatase PP2A, followed by recruitment of a member of the c-Src family tyrosine kinases, usually pp60c-src, to the PyMT/PP2A complex (3). Binding to PyMT/PP2A promotes c-Src “open” conformation and autophosphorylation of its Tyr416, resulting in activating its kinase activity and in phosphorylating PyMT tyrosines. Three of these phosphotyrosines act as binding sites for the SH2 or PTB domains of ShcA (PyMT(Tyr250)), PI3K (PyMT(Tyr315)) and PLCγ (PyMT(Tyr322)), resulting in activating the Ras/Raf/MEK/MAPK, PI3K/PDK1/Akt, and PLCγ/PKC survival pathways, respectively (1). Association of the PyMT/PP2A/c-Src complex with the plasma membrane is required for its transforming activity (4), implying that the complex may be visualized as an oncogenic RTK, with c-Src acting as its obligatory tyrosine kinase. In addition to c-Src, MMTV-PyMT breast cancer is driven by STAT3 (5), being activated by phosphorylation of its Tyr705 by activated c-Src ((5), (6), (7)) or JAK (8). Indeed, de-phosphorylation of the gp130/JAK
complex by the tyrosine phosphatase Shp2 is reported to result in suppressing PyMT-induced transformation, while knocking down Shp2 potentiates PyMT tumorigenesis (9). The c-Src/STAT3 interplay that drives PyMT-induced transformation is similar to that previously reported for the large tumor antigen of the Simian virus 40 (10, 11). Since 70% of human breast cancers express c-Src (12), while 50% express constitutive STAT3 (13), treatment strategies for suppressing the c-Src and STAT3 oncogenes of MMTV-PyMT breast cancer may be of relevance in treating human breast cancer (14).

Carbohydrate-restricted ketogenic diets, enriched in fat at the expense of carbohydrates, have been repeatedly reported to suppress breast cancer ((15), (16) and References therein). Tumor suppression by ketogenic diets has been ascribed to limiting insulin and IGF1 acting as growth factors (reviewed in (17)), and to the obligatory requirement of malignant cells for glucose-derived metabolites (e.g., nucleotides, NADPH) for supplying their biomass demands (Warburg effect, reviewed in (18)). Indeed, breast cancer is promoted by insulin resistance / hyperinsulinemia, and insulin sensitizers used for treating type 2 diabetes (e.g., metformin) appear to be effective in suppressing tumorigenesis in general and breast cancer in particular (19). Alternatively, the efficacy of ketogenic diets in suppressing tumorigenesis may further be ascribed to the inherent efficacy of free / nonesterified long-chain fatty acids (LCFA), if allowed to reach high enough intra-cellular concentrations. Carbohydrate restriction may indeed allow for that, by limiting the availability of glycerol-3-phosphate and insulin required for the esterification of LCFA into downstream lipid products (20).

The putative inherent efficacy of non-esterified LCFA to suppress transformation may be simulated by MEDICA analogs. MEDICA analogs (21) consist of long-chain,
\(\alpha,\omega\)-dioic acids \([\text{HOOC-C(\(\alpha'\))-C(\(\beta'\))-Q-C(\(\beta\))-C(\(\alpha\))-COOH}\], where Q represents a long-chain core element], substituted in the \(\alpha\alpha'\) (M\(\alpha\alpha\)), \(\beta\beta'\) (M\(\beta\beta\)), and/or other optional core carbons. MEDICA analogs may be thioesterified to their respective CoA-thioesters, but these are not esterified into lipids, nor converted into ceramides, while the substitutions at the \(\alpha\alpha'\) or \(\beta\beta'\) positions block their \(\beta\)-oxidation. MEDICA analogs are mostly excreted in bile as respective glucuronides. As such, MEDICA analogs may mimic allosteric activities of free / non-esterified LCFA, while avoiding their role as substrates for \(\beta\)-oxidation or esterification into lipids. Moreover, MEDICA analogs proved anti-diabetic hypolipidemic efficacy in a series of obese diabetic animal models (22-24,26), prompting our interest in studying the efficacy of ketogenic diet and MEDICA in the breast cancer context.

**Methods**

*Animals and diets:* FVB wild-type females (Harlan Inc. Jerusalem, Israel) were bred in-house with FVB MMTV-PyMT heterozygous males (Dr. Itay Ben-Porat, Hebrew University Medical School). F1-generation females were genotyped by PCR using the PyMT primers: Fw: 5' GGAAAGTCACTAGGAGG-AGGG 3'; Rev: 5' GGAAGCAAGTACTTGAGG-AGGG 3'. Mice were kept in standard SPF conditions in 12 hours light/dark periods, with free access to food and water. Mice were fed with Teklad 2018S standard rodent diet (54% carbohydrate, 18% fat, 24% protein energy), or with Teklad 93075 high fat diet (HFD) (24% carbohydrate, 54.8% fat, 21.2% protein energy), or with Teklad 96355 ketogenic diet (0.4% carbohydrate, 90.5% fat, 9.1% protein energy). Teklad 96355 diet was freshly prepared as gelatin chips every 3 days.
The MEDICA diet consisted of 0.04% (w/w) MEDICA \([\text{HOOC-}C(\text{CH}_3)_2-\text{CH}_2]_{12}-C(\text{CH}_3)_2-\text{COOH}\] mixed in Teklad 2018S standard rodent diet (yielding a dose of 40mg MEDICA/kg body weight/day). Energy intake was determined by measuring food consumption every three days. 2h prior to sacrifice mice were injected with BrdU i.p where indicated. Upon sacrifice, mice were anesthetized using 85% Ketamine/15% Xylazine (0.1 ml per 25–30 gram body weight). Breast tumors and lungs were sampled in buffered 4% formaldehyde for further histochemistry/immunohistochemistry analysis, or in liquid nitrogen for RNA and protein analysis. Animal care and experimental procedures were in accordance with the accredited animal ethics committee of the Hebrew University Medical School.

**Immunohistochemistry:** Mammary tumor specimens fixed in 4% buffered formaldehyde were embedded in paraffin. 5µm sections were de-waxed and re-hydrated through graded ethanol dilutions, followed by cooking in 25mM citrate buffer, pH 7.4, at 115°C for 3 minutes. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Sections were then incubated with the primary antibodies as indicated, followed by incubation with horseradish peroxidase (HRP) or fluorescent-conjugated secondary antibodies (Jackson Laboratories). HRP-incubated sections were counterstained with hematoxylin. Fluorescent intensity was analyzed by confocal microscopy using DAPI for nuclei visualization (Zeiss LSM 710, Axio observer Z1). Lungs were excised and fixed in formalin overnight and then paraffin-embedded. 5µm sections were stained with H&E and analyzed using computerized microscopy (Ariol SL-50, Olympus BX61 Applied Imaging, CA).
**Cell cultures:** MMTV-PyMT primary cells were isolated from 16-week old MMTV-PyMT mice as described (25). Met-1 cells (Dr. Alexander Borowsky, Center for Comparative Medicine, UC Davis) and primary cells were cultured in complete DMEM (Biological Industries, Beit Haemek, Israel) supplemented with Penicillin-Streptomycin solution, L-glutamine and 10% fetal calf serum, followed by adding MEDICA as indicated. Cell proliferation was quantified using the Methylene Blue assay. Met-1 cells cultured in 24 plate were transfected with M67-TATA-TK-LUC (1\(\mu\)g) reporter plasmid, expression plasmids for constitutive STAT3 (0.1\(\mu\)g) and for CMV-\(\beta\)-galactosidase (0.1\(\mu\)g), using jetPEI DNA transfection reagent (26). Following 8h, medium was changed and cells were treated for 24h with MEDICA as indicated. Luciferase activity normalized to \(\beta\)-galactosidase was measured as previously described (26). HCC1954 cells (ATCC CRL-2338) were cultured in RPMI1640 (Biological Industries, Beit Haemek, Israel) supplemented with Penicillin-Streptomycin solution and 10% fetal calf serum, and added MEDICA as indicated. Where indicated, HCC1954 cells were infected with Lentivirus knockout constructs of sh-e-Src, sh-STAT3 or scramble plasmids (Sigma Mission), followed by puromycin selection.

**Clonogenic assay:** Cells of respective experimental plates were trypsinized. 7500 cells were plated on 60mm plate and allowed to form colonies for 10 days. Cells were fixed with 0.625% gluteraldehyde and stained with methylene blue. Colonies exceeding 400 microns in size were counted.

**Cell lysis and Western blotting:** Cultured cells were scraped with 1X lysis buffer (50mM Tris HCl pH 8.0 ,1% Triton X-100 ,1mM EGTA ,1mM EDTA ,150mM NaCl, 5mM NaPPI ,50mM NaF ,1mM PMSF, 1mM Na Vanadate ,40nM bpVfan and protease
inhibitor cocktail (Sigma), and centrifuged for 15 min at 12,500 rpm. Frozen tumor samples were homogenized in lysis buffer using Polytron homogenizer. Protein concentration was determined by BCA (Thermo Scientific). Unless otherwise indicated, protein lysates were prepared in SDS sample buffer (62mM Tris (pH 6.8), 2.3% SDS, 0.64mM mercaptoethanol, 10% glycerol), subjected to SDS-PAGE, electrotransferred onto cellulose nitrate membranes (Schleicher & Schuell, Dassel, Germany) and probed with the indicated first antibody, followed by HRP-labeled second antibody. Where indicated, protein lysates were prepared in SDS sample buffer lacking mercaptoethanol. Bands were detected by ECL, and the intensity of individual bands was determined by densitometry using TINA 2.10 software.

**Immunoprecipitation:** MET-1 cells were incubated as indicated. Following incubation, cells were rinsed once in cold PBS and lysed with 50mM Hepes (pH 7.4), 150mM NaCl, 10% Glycerol, 1.5mM MgCl2, 1mM EGTA, 1% Triton, 50mM β-glycerolphosphate, 25mM NaF, 1mM Na-vanadate, 40nM bpVphen, protease inhibitor mix (Sigma) and 17.5 mg/ml Octyl beta-D-glucopyranoside (Sigma). Lysates were kept on ice for 30 min and centrifuged at 12000 rpm for 15min. 250µg of lysate were incubated for 4h with protein A/G beads (Santa Cruz Biotechnology) pre-loaded with the indicated antibody. The immunoprecipitate was rinsed 3 times with washing buffer (20mM Hepes (pH 7.4), 150mM NaCl, 0.1%Tx-100, 10% Glycerol), suspended in SDS sample buffer and boiled for 8min. Immunoprecipitated proteins were analyzed by SDS-PAGE/Western blotting.

**qRT-PCR:** RNA was purified from frozen mouse tissues using the Total RNA Mini Kit (Geneaid). 0.5µg of RNA were used as template for cDNA synthesis using M-MLV reverse transcriptase (Invitrogen). Real Time PCR was carried out (Rotorgene, Corbett
Research) using KAPA SYBR FAST qPCR MIX (Kapa Biosystems) with the following primers (5’ to 3’): MMP9 (Fw: AACCTCCAACCTCACGGACAC, Rev: CTGCT-TCTCTCCCATCATCTGG); E-cadherin (Fw: TCATCATTGAGAGGGAGACGGCT, Rev: TGGGTAAACTCTGGCCTGGTGTCA); Ezh2 (Fw: GACGATGATGGAGATGTCCAGATG, Rev: CCGAG-GTGGGCAA GTTTCTTTATC); PyMT (Fw: CTCCAACAGATCACCACGCACAC, Rev: GCTGGTCTTGGTCGCTTTTCTGGA-TAC). mRNA was quantified using the delta delta Ct method (27).

**ELISA:** VEGF content of mouse lysates was determined using Quantikine mouse VEGF kit (R&D System, Minneapolis) according to manufacturer instructions.

**ROS production:** ROS production was determined by 2,7 dichlorofluoresceine diacetate (DCF) (5 μM) added to respective cell cultures for the last 15 min of incubation. Cells were washed once with PBS, and lysed with 0.5% TX-100. DCF fluorescence was determined by 485/530 nm excitation/emission analysis. Hydrogen peroxide production was determined by incubating respective growth media (phenol red-free) with HRP / Amplex Red (Invitrogen). Fluorescence was determined by 560/590 nm excitation/emission analysis. Glutathione (GSH)/Glutathione disulfide (GSSG) ratio was determined by the GSH/GSSG-Glo assay (Promega) according to manufacturer protocol.

**Antibodies:** Anti-β-casein, anti-Erk and anti-phospho-Erk( Tyr204) antibodies were from Santa Cruz biotechnology; anti-pHH3, anti-Akt, anti-phospho-Akt(Ser473), anti-phospho-c-Src(Tyr416), anti-phospho-c-Src(Tyr527), anti-cleaved caspase-3, anti-phospho-FAK(Tyr925), anti-phospho-p130CAS(Tyr910) antibodies were from Cell Signaling; anti-c-Src and anti-Ezh2 antibodies were from Millipore; anti-BrdU antibody was from Thermo Scientific; anti-CD34 antibody was from Cedarlane; anti-E-cadherin,
anti-β-catenin, anti-p130CAS and anti-FAK antibodies were from BD Transduction Laboratories; anti-tubulin antibody was from Sigma. c-Src kinase inhibitors were from LC Laboratories.

Statistics: Statistics was performed by two-tailed homoscedastic repeated measure analysis of variance. Significance was analyzed by paired t test.

Results

Suppression of MMTV-PyMT tumor growth by carbohydrate restriction

Suppression of MMTV-PyMT tumorigenesis by ketogenic diet has been verified in transgenic FVB MMTV-PyMT mice fed throughout weeks 4 to 12 with either standard, high fat or ketogenic diet, consisting of 3.2, 0.4 and 0.004 carbohydrate/fat energy ratios, respectively. Tumor mass decreased by 65% upon increasing dietary fat energy at the expense of dietary carbohydrate (Fig 1A), with increase in tumor latency (not shown). Suppression of tumor mass by ketogenic diet was accompanied by decrease in BrdU staining (Figure 1B). The extent of decrease in tumor mass by ketogenic diet was essentially similar to that of MEDICA dosed with standard carbohydrate-rich diet (Fig 1A).

Suppression of MMTV-PyMT tumorigenesis by ketogenic diet was not accounted for by caloric restriction that may accompany use of ketogenic diets (15). Indeed, daily energy consumption amounted to 9.3, 10.2, 16.0 and 14.1 kcal/mouse for the standard, high fat, ketogenic diet and MEDICA feed, respectively. The 150-170% increase in ketogenic energy consumption did not result in any significant change in body weight gain (23.4±2.3, 22.5±1.3 and 23.5±2.4 gr for 12 week control, ketogenic and MEDICA mice,
respectively). In the absence of visible diarrhea or steatorrhea, the increase in energy consumption may imply an increase in total body energy turnover of MMTV-PyMT mice maintained on ketogenic diet or MEDICA feed.

**Suppression of MMTV-PyMT tumorigenesis by MEDICA**

MEDICA treatment of transgenic FVB MMTV-PyMT female mice throughout weeks 4-12 or weeks 4-18 (denoted by 4/12 and 4/18, respectively) resulted in 75% decrease in tumor mass as compared with control mice (Fig 2A). Suppression of tumor growth was also reflected in longer tumor latency in MEDICA-treated mice, where palpable tumors of MEDICA-treated mice were delayed by two weeks as compared with control mice (10 and 8 weeks, respectively). Moreover, MEDICA treatment throughout weeks 12-18 (denoted by 12/18) resulted in robust decrease in tumor mass (Fig 2A), implying growth suppression of established MMTV-PyMT tumors.

Non-treated tumors consisted of highly-dense poorly-differentiated mammary carcinoma, whereas MEDICA-treated tumors consisted of areas of papillary glandular structures, with occasional cysts lined by squamous epithelium and filled with fluid containing casein (Figs 2B, C), implying more differentiated morphological pattern.

Most importantly, MEDICA treatment resulted in robust decrease in lung metastases (Fig 2D). Thus, the number of lung metastatic foci, and in particular the percentage of lung area occupied by lung metastases, were significantly decreased by MEDICA (12/18) treatment (5.9% vs. 0.4% lung area in non-treated and MEDICA-treated mice, respectively), implying growth inhibition of established lung metastatic foci.

Suppression of MMTV-PyMT tumor growth by MEDICA was not accounted for by loss of the PyMT transgene, or by interfering with the overall hormonal balance of treated
mice. Thus, PyMT expression remained unaffected by MEDICA treatment, and MEDICA-treated female mice were fertile (not shown).

Suppression of MMTV-PyMT tumor growth by MEDICA was further evaluated in terms of proliferation, apoptosis and angiogenesis. MEDICA treatment resulted in pronounced decrease in BrdU incorporation, being sparse and mainly evident at the periphery of tumor lesions (Fig 3A). In line with decreased BrdU incorporation, MEDICA treatment resulted in 75% decrease in tumor mitotic index verified by p-HH3 staining (Fig 3B). Inhibition of proliferation by MEDICA was accompanied by increase in apoptosis, verified by cleaved caspase-3 staining (Fig 3C). Increased apoptosis was mainly evident in tumor samples of MEDICA (4/18), whereas decreased proliferation was mostly pronounced in tumor samples of MEDICA (4/12). Suppression of MMTV-PyMT tumor growth by MEDICA was further accompanied by decrease in tumor vascularization as verified by decrease in the CD34 endothelial marker (Figure 3D) and tumor VEGF content (Fig 3E).

**MMTV-PyMT mesenchymal-epithelial transition (MET) in response to MEDICA**

The higher epithelial differentiation pattern induced by MEDICA, combined with inhibition of MMTV-PyMT metastasis, was further evaluated by verifying epithelial-mesenchymal biomarkers in response to MEDICA (Fig 4). MEDICA treatment resulted in increase in E-cadherin expression (Fig 4A), being accompanied by increase in membranous β-catenin (Fig 4B), with pronounced decrease in MMP9 expression (Fig 4C). MEDICA-induced expression of E-cadherin combined with suppression of MMP9 expression was further evaluated in terms of transcription factors that may control MET. Ezh2 (polycomb group protein enhancer of zeste homolog 2) is the histone
methyltransferase catalytic subunit of the polycomb repressive complex 2 (PRC2), being overexpressed in a variety of aggressive breast cancers (28, 29). Ezh2 is associated with genome instability, disruption of mammary ductal morphogenesis (30), and invasion and metastasis of malignant breast cancer, being partly accounted for by suppression of E-cadherin and induction of MMPs expression ((31, 32). Ezh2 transcript, protein and cell content were suppressed by MEDICA (Fig 4D), pointing to mammary epithelial transition of MEDICA-treated MMTV-PyMT tumors.

**Suppression of PyMT c-Src and STAT3 oncogenic drivers by MEDICA**

Suppression of the MMTV-PyMT oncogenic drivers by MEDICA was evaluated in Met-1 (33) and primary MMTV-PyMT cells. MEDICA inhibited the proliferation of primary MMTV-PyMT and of Met-1 cells (Fig 5A). The µM concentrations of MEDICA reflect the high binding affinity of MEDICA analogs to serum albumin (higher than 99%, independently of MEDICA concentrations in the range of 0-0.9 mM (not shown)), resulting in nM concentrations of the free MEDICA acid in the culture medium.

The roles played by the c-Src and STAT3 transduction pathways in PyMT cell survival were verified in Met-1 cells. In line with the transforming role played by c-Src / PyMT, cell growth was inhibited by the c-Src kinase inhibitor dasatinib (34) (Supp Fig 1A). However, cell growth was only partially suppressed by dasatinib under saturating dasatinib concentrations, whereby phospho-c-Src(Tyr416) was 90% inhibited, implying the activity of additional oncogenic driver(s). Indeed, Met-1 cell growth was inhibited by the JAK / STAT3(Tyr705) inhibitor pyridine 6 (P6) (13) (Supp Fig 1B), indicating that STAT3 serves as oncogenic driver for PyMT cell survival. However, here again suppression of Met-1 cell growth by P6 was only partial, whereby phospho-
STAT3(Tyr705) was essentially abrogated, implying that Met-1 survival is driven by each of the c-Src and STAT3 oncogenes. Moreover, phospho-STAT3(Tyr705) of Met-1 cells was only marginally (<20%) inhibited by the c-Src inhibitor dasatinib (not shown), while phospho-c-Src(Tyr416) was not affected by the JAK inhibitor P6, indicating that the c-Src and STAT3 transduction pathways act as independent oncogenic drivers. In line with that, inhibition of Met-1 cell growth by combining dasatinib and P6 was additive (Supp Fig 1C), implying that Met-1 survival is independently driven by both, c-Src and STAT3.

In line with our previous findings in other cell types (26), MEDICA treatment resulted in suppressing STAT3(Tyr705) and Akt(Ser473) phosphorylation in primary MMTV-PyMT cells, and in suppressing the transcriptional activity of STAT3 in Met-1 cells (Fig 5B), indicating that suppression of PyMT survival by MEDICA may partly be ascribed to inhibition of the STAT3 transduction pathway. MEDICA efficacy in suppressing c-Src/PyMT was pursued by analyzing the c-Src content in PyMT immunoprecipitates of Met-1 cells. MEDICA treatment resulted in abrogating c-Src association with PyMT (Fig 5C), implying suppression of c-Src-induced PyMT transduction. MEDICA apparent efficacy in inducing a conformational change in c-Src was further indicated by probing c-Src sensitivity to dasatinib in the absence and presence of added MEDICA. Indeed, dasatinib concentrations that were only partly effective in inhibiting phospho-Src(Tyr416), became fully effective by added MEDICA, implying an apparent MEDICA-induced increase in c-Src affinity for dasatinib (Fig 5D). MEDICA-induced increase in c-Src affinity for dasatinib was further verified by the combined effect of MEDICA and dasatinib in suppressing Met-1 cell growth, under conditions of short incubation time.
(24h) whereby MEDICA stand-alone was essentially ineffective in suppressing cell growth. Added MEDICA resulted in synergizing dasatinib activity in suppressing Met-1 cell growth by dasatinib concentrations that were only partially effective in the absence of MEDICA (Fig 5E). That is in contrast to P6, where its combination with dasatinib was strictly additive (Supp Fig 1C). The mode of action of MEDICA in suppressing c-Src transforming activity has been further studied in human HCC1954 breast cancer cells (overexpressing ErbB2, ER- PR-negative).

**Suppression of human HCC1954 breast cancer cell growth by MEDICA**

HCC1954 growth was inhibited by shc-Src, dasatinib, shSTAT3 and P6 (Supp Fig 2), indicating that both, c-Src and STAT3 drive oncogenesis in this cell line. Also, dasatinib failed to suppress STAT3(Tyr705) phosphorylation, while P6 failed to suppress c-Src(Tyr416) phosphorylation (not shown), indicating that, similarly to PyMT cells, HCC1954 cell survival was independently driven by the two concerned oncogenes.

MEDICA treatment suppressed HCC1954 cell growth and clonogenity (Fig 6A). Similarly to MEDICA effects in primary PyMT and in Met-1 cells (Fig 5B), growth inhibition of HCC1954 cells was accompanied by abrogating the STAT3 and Akt transduction pathways (Fig 6B). Also, MEDICA efficacy in suppressing growth of HCC1954 cells was partly abrogated in cells infected with shc-Src (Fig 6B), implying c-Src as MEDICA target in suppressing HCC1954 growth.

Similarly to abrogating the c-Src/PyMT association in Met-1 cells (Fig 5C), MEDICA treatment of HCC1954 cells resulted in increase in c-Src oligomers and other non-identified high molecular weight adducts, with concomitant decrease in the c-Src monomers, exemplified by SDSPAGE under non-reducing conditions (Fig 6C). In line
with that, and in apparent similarity to classical c-Src kinase inhibitors, MEDICA-induced c-Src oligomerization resulted in suppressing the phosphorylation of downstream substrates of c-Src, like FAK(Tyr925) or p130CAS(Tyr410) (Fig 6D). Surprisingly however, decrease in c-Src monomers and its activity by MEDICA treatment was accompanied by MEDICA-induced increase in Tyr416 phosphorylation of c-Src monomers and oligomers (Figs 6E), implying that c-Src conformational change induced by MEDICA may account for its inactivation, while paradoxically increasing its Tyr416 phosphorylation.

Cysteine oxidation of c-Src by reactive oxygen species (ROS) (e.g., O$_2^-$, H$_2$O$_2$) has recently been reported to result in c-Src homodimerization (35) and in autophosphorylating its Tyr416 (36, 37), while activating (36) or inhibiting (35, 38) its kinase activity. Indeed, MEDICA treatment of HCC1954 cells resulted in increase in ROS production and H2O2 release into the culture medium, with concomitant decrease in GSH/GSSG ratio (Fig 6F). ROS production by MEDICA was similarly observed in Met-1 cells, being abrogated by added N-acetyl cysteine (NAC) (not shown). MEDICA-induced c-Src oligomerization and c-Src(Tyr416) autophosphorylation were all abrogated by added NAC (Fig 6G), implying that c-Src oxidation by MEDICA-induced ROS may account for suppressing its activity in the breast cancer context. Also, suppression of HCC1954 clonogenity by MEDICA was partly abrogated by added PEG-SOD (Fig 6H), implying that MEDICA-induced ROS may partly account for growth inhibition of breast cancer cells. Moreover, ketogenic diets were reported to sensitize lung cancer cells to radiation-induced cell killing by a mechanism that appeared to involve oxidative stress (39). In mimicking that efficacy of ketogenic diets, pretreatment of HCC1954 cells with
MEDICA, followed by their irradiation, resulted in amplified suppression of their clonogenity (Supp Fig S3), implying the prospective efficacy of MEDICA in sensitizing tumor cells to radiation treatment.

**Discussion**

70% of human breast cancers express active c-Src, while 50% express constitutive STAT3 (12, 13). Furthermore, c-Src is reported to be a common oncogenic driver of variable trastuzumab resistance pathways, implying that suppressing its activity may overcome resistance (40). Similarly, STAT3 plays an essential role in breast cancer stem cells, being correlated with tamoxifen resistance (41). Hence, treatment strategies for suppressing the c-Src / STAT3 interplay are of relevance in treating human breast cancer (14).

c-Src plays an obligatory role in inducing breast cancer in MMTV-PyMT transgenic mice, where membranous PyMT phosphorylation by c-Src results in cell proliferation and survival (42). Hence, the c-Src / PyMT complex may be visualized as oncogenic human RTK, with c-Src acting as its obligatory tyrosine kinase. Concomitantly, PyMT cell survival is promoted by constitutive STAT3 (5). Similarly to the MMTV-PyMT model, survival of human HCC1954 breast cancer cells is shown here to be driven by both, c-Src and STAT3. Of note, the c-Src and STAT3 oncogenic drivers are shown here to act independently in promoting MMTV-PyMT as well as HCC1954 cell survival, underscoring the need for abrogating both in order to suppress breast cancer. Suppression of MMTV-PyMT and HCC1954 cell survival by MEDICA is shown here to be partly accounted for by suppressing the STAT3 transduction pathway, in line with our
previous reports in other cell types (26). Suppression of STAT3 in MMTV-PyMT and HCC1954 cells by MEDICA is reflected by decrease in phospho-STAT3(Tyr705) and in STAT3 transcriptional activity. Suppression of cell survival by MEDICA is further due to MEDICA-induced ROS production, resulting in inactive c-Src that fails to activate its downstream substrates (35, 38). Thus, MEDICA treatment is shown here to abrogate c-Src association with its PyMT scaffold, as well as to suppress the phosphorylation of c-Src downstream substrates like FAK(Ser925) and p130CAS(Tyr410) in HCC1954 cells. That is in spite of enhancing c-Src(Tyr416) autophosphorylation, implying a mode of inhibition of c-Src transforming activity that differs from that of classical c-Src kinase inhibitors (e.g., dasatinib, saracatinib, bosutinib (43)).

c-Src oxidation has been reported to result in its heterodimerization and inactivation (35), while others have reported increase in c-Src Tyr416 phosphorylation, resulting in enhancing its kinase activity, tumorigenesis and metastasis (36, 37, 44). Increase in c-Src(Tyr416) autophosphorylation and enhanced kinase activity has been ascribed to c-Src oxidation that results in a covalent S-S bond between its SH2 Cys245 and Cys487 of its kinase domain (37). In contrast, oxidation of Cys277 in c-Src GQGCFG glycine loop is reported to result in c-Src oligomerization and loss of c-Src activity (35), due presumably to decrease in c-Src affinity for its downstream substrates. Of note, oxidation of c-Src by MEDICA-induced ROS is shown here to result in abrogating c-Src kinase and transforming activity, while concomitantly increasing its Tyr416 phosphorylation, implying that the two oxidative modes of modulating c-Src activity are not mutually exclusive. Indeed, concomitant c-Src oxidation by the two oxidative modes may result in
inactive c-Src oligomers having an “open” conformation with increase in c-Src(Tyr416) autophosphorylation (Figs 6D, G).

Suppression of MMTV-PyMT tumor by MEDICA resulted in suppressing tumor mass, proliferation, mitotic index and angiogenesis, while promoting tumor apoptosis. Most importantly, suppression of tumor survival was accompanied by increase in breast markers of differentiation, as evident by tumors consisting of papillary glandular structures, with cysts lined by squamous epithelium filled with fluid containing casein. Similar differentiation profile has previously been reported in MMTV-PyMT transgenic mice treated with the c-Src kinase inhibitor bosutinib (SKI606) (45) as well as in c-Src null mice (42), implying that inhibiting c-Src activity by kinase inhibitors, genetically, or by its oxidation may converge onto a similar phenotype.

Suppression of MMTV-PyMT tumor growth by MEDICA is further shown here to be accompanied by suppressing lung metastasis. Suppression of lung metastasis may be ascribed to suppressing the primary tumor, resulting in decreased probability for tumor dissemination, complemented by inhibiting epithelial-mesenchymal transition (EMT) of tumor cells. Indeed, MEDICA treatment was found to induce the enrichment of tumor cells with membranous E-cadherin and β-catenin, accompanied by decrease in MMP9 transcript. Since both, STAT3 and c-Src are potent inducers of EMT (46, 47), tumor cell epithelialization by MEDICA may partly be ascribed to abrogating EMT. EMT suppression by MEDICA may further be partly accounted for by suppressing Ezh2 transcription. Since Ezh2 is involved in controlling cell proliferation, differentiation and apoptosis (31, 32), its suppression by MEDICA may further account for suppressing tumor growth while promoting tumor differentiation.
Suppression of MMTV-PyMT tumor growth by MEDICA apparently simulates that of ketogenic diet, indicating that the efficacy of carbohydrate-restricted/ketogenic diets in suppressing tumorigenesis may perhaps reflect the inherent tumor-suppressive efficacy of free LCFA or their respective CoA-thioesters, if allowed to reach high enough intracellular concentrations, by suppressing their esterification into lipids due to limiting insulin and glycerol-3-phosphate. The apparent similar phenotypes induced by MEDICA treatment and ketogenic diets may indicate that both may converge to the same target(s). Indeed, the induced increase in total body energy turnover by the two concerned effectors may perhaps point to mitochondria being the target of both effectors. Indeed, LCFA as well as MEDICA compounds have been previously reported to induce low-conductance gating of the mitochondrial permeability transition pore (PTP) (48, 49). PTP gating results in robust increase in oxygen consumption due to decrease in inner mitochondrial membrane potential, and in increase in mitochondrial ROS production due to loss of mitochondrial reductive components (50). Since the compliance to ketogenic diets is quite poor, MEDICA treatment may offer the benefit of simulating carbohydrate restriction while maintaining a balanced diet.

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Author contribution
Study conception and design: JBT, UG, RH; Acquisition of data: UG, RH, MS, ES, SO; Analysis and interpretation of data: UG, RH, EP, JBT; Drafting of manuscript: JBT; Critical revision: UG, RH, EP
References

Legends to Figures

Figure 1. Suppression of MMTV-PyMT tumorigenesis by ketogenic diet.
A. MMTV-PyMT female mice were fed ad libitum from weaning (4 weeks of age) to 12 weeks with standard rodent diet (Control), high fat diet (HFD) (Teklad 93075), ketogenic diet (Keto) (Teklad 96355) or standard rodent diet mixed with MEDICA. Upon sacrifice, breast tumors were dissected and weighed to yield total body tumor mass. Numbers denote respective dietary carbohydrates/fat energy ratio. Mean ± SE (n=8-10 mice per group) *- Significant as compared to control diet (p< 0.05). **- Significant as compared to control diet (p< 0.002).  B. Representative photomicrographs (X20) of MMTV-PyMT breast tumors of mice maintained on standard (Control) or ketogenic (Keto) diet throughout weeks 4-12. Sections were stained with H&E and immunostained with BrdU as described in Methods.

Figure 2. Suppression of MMTV-PyMT tumorigenesis by MEDICA.
MMTV-PyMT female mice were fed ad libitum for 12 weeks (control (12)) (n = 8) or 18 weeks (control (18)) (n = 9) with standard rodent diet (Teklad 2018) in the absence or presence of added MEDICA (0.04% w/w), from week 4 to week 12 (MEDICA (4/12)) (n = 8), week 4 to week 18 (MEDICA (4/18)) (n = 8), or week 12 to week 18 (MEDICA (12/18)) (n = 5) as indicated.  A. Upon sacrifice, breast tumors were dissected and weighed to yield total tumor mass. Points represent total tumor mass of individual mice; lines denote mean value of each group. *- Significant as compared to respective control (p < 0.001).  B. Representative H&E-stained photomicrographs of MMTV-PyMT tumors of control, MEDICA (4/12) and (4/18)-treated MMTV-PyMT mice. Note the cystic (black arrows), papillary (red arrows) and squamous-like (blue arrows) structures.  C. Representative β-casein-immunostained photomicrographs of control and MEDICA (4/12) tumors.  D. Representative H&E-stained lung photomicrographs of control and MEDICA (12/18)-treated MMTV-PyMT mice. Metastasis appears as dark spots. Number of metastatic foci and the percentage fraction of metastatic area per lung were determined by computerized quantitative histology of respective H&E-stained lung
photomicrographs. Points represent values of individual mice; Lines denote median value of each group. *- Significant as compared to control (p< 0.05).

Figure 3. MMTV-PyMT tumor proliferation, apoptosis and angiogenesis in response to MEDICA.
MMTV-PyMT female mice were fed ad libitum with standard rodent diet (Telkad 2018) in the absence or presence of added MEDICA (0.04% w/w), from week 4 to week 12 (4/12) or week 4 to week 18 (4/18) as indicated. A. Representative BrdU-immunostained photomicrographs of MMTV-PyMT tumors of control, MEDICA (4/12)- and MEDICA (4/18)-treated mice. Cysts are stained in brown. B. Representative P-HH3-immunostained photomicrographs of MMTV-PyMT tumors of control and MEDICA (4/12)-treated mice. Mitotic index was determined by counting the number of p-HH3-stained cells of 10 fields/mouse. Mean ± SE (n= 8). *- Significant as compared to control (p< 0.05). C. Representative photomicrographs of cleaved-caspase3-immunostaining of MMTV-PyMT tumors of control and MEDICA (4/18)-treated mice. Apoptotic index was determined by counting the number of cleaved caspase3-stained cells of 10 fields/mouse. Mean ± SE (n=6). *- Significant as compared to control (p< 0.05). D. Representative CD34-immunostained photomicrographs of MMTV-PyMT tumors of control and MEDICA (4/18)-treated mice. Total CD34-stained area was determined by screening the CD34-stained area of 10 fields/mouse, using computerized microscopy. *- Significant as compared to control (p< 0.05). E. VEGF content of MMTV-PyMT tumors of control and MEDICA-treated mice. VEGF content was determined by ELISA in tumor lysates of control, MEDICA (4/12) and MEDICA (4/18)-treated mice. VEGF content of 12-week control is defined as 1.0. Mean ± SE (n=8). *- Significant as compared to the respective control (p< 0.001).

Figure 4. MMTV-PyMT mesenchymal-epithelial transition (MET) in response to MEDICA
MMTV-PyMT female mice were fed ad libitum with standard rodent diet (Telkad 2018) in the absence or presence of added MEDICA (0.04% w/w), from week 4 to week 12 (4/12). Representative immunostained photomicrographs of E-cadherin (A), β-catenin (B)
and EZH2 (D) of MMTV-PyMT tumors of control and MEDICA(4/12)-treated mice. Total membranous β-catenin-stained area was determined by screening the β-catenin-stained area of 10 field/mouse, using computerized microscopy. E-cadherin (A), EZH2 (D) and MMP9 (C) transcripts were determined in respective tumor samples by qRT-PCR as described in Methods, using HPRT1 as reference. E-cadherin (A) and EZH2 (D) protein content normalized by tubulin was determined in respective tumor lysates by Western blotting as described in Methods. Mean ± SE (n=6-8). *- Significant as compared to control (p<0.05).

Figure 5. Suppression of MMTV-PyMT c-Src and STAT3 transduction by MEDICA.

A. Primary MMTV-PyMT cells and Met-1 cells were cultured in the presence of MEDICA as indicated. Cell growth was measured by the methylene blue method. Cell density on day 0 is defined as 1.0. Representative experiment.  B. Left frame: Met-1 cells were transfected with STAT3 reporter plasmid and constitutive STAT3 expression plasmid, followed by incubating the cells for 24h in the absence or presence of 200μM MEDICA as described in Methods. Luciferase activity of control cells is defined as 1.0. Mean ± SE of three independent experiments. *- Significant as compared to control (p<0.05). Right frame: Primary MMTV-PyMT cells were cultured for 24h in the absence or presence of 200μM MEDICA. Tumor lysates were subjected to SDS-PAGE as described in Methods. Representative experiment of three different preparations of primary MMTV-PyMT cells.

C. Met-1 cells were cultured in the absence or presence of 200μM MEDICA for 12 and 24h as indicated. Cell lysates were immunoprecipitated by anti-PyMT antibody, followed by immunoblotting with anti-PyMT or anti-c-Src antibody as indicated. The lower panel shows c-Src input. Representative experiment.

D. Met-1 cells were treated with 200μM MEDICA for 24h, followed by added dasatinib as indicated for the last 2h. Cell lysates were subjected to SDS-PAGE and analyzed by immunoblot for phospho-c-Src(Tyr416) as described in Methods. Representative experiment.

E. Met-1 cells were grown for 24h with increasing concentrations of dasatinib as indicated in the presence or absence of 200 μΜ MEDICA.
added 1h before Dasatinib. Cell growth was measured by the methylene blue method. Cell density without additions is defined as 1.0. Representative experiment.

**Figure 6. Suppression of HCC1954 tumorigenesis by MEDICA**

**A.** *Upper frame:* HCC1954 cells were cultured in the presence of MEDICA as indicated. Cell growth was measured by the methylene blue method. Cell density without addition is defined as 1.0. Representative experiment. *Lower frame:* HCC1954 cells were cultured for 24h in the presence of MEDICA as indicated. Cells were trypsinized and subjected to clonogenic assay. Colonies exceeding 400 microns in size were counted. Representative images.  

**B.** *Left:* HCC1954 cells were cultured for 24h in the presence of 200µM MEDICA. Cell lysates were subjected to SDS-PAGE as described in Methods. Representative experiment. *Right:* HCC1954 cells were infected with sh-c-Src or sh-scramble as indicated, followed by culturing the cells for 48h with MEDICA as indicated. Cell growth was measured by the methylene blue method. Respective cell densities in the absence of added MEDICA are defined as 1.0. Representative experiment.  

**C.** HCC1954 cells were treated for 24h with MEDICA as indicated. Cell lysates were prepared in SDS sample buffer with or without mercaptoethanol, subjected to SDSPAGE and analyzed by immunoblot for c-Src monomers and oligomers.  

**D.** HCC1954 cells were treated for 24h with 200µM MEDICA, or for 2h with 0.2 µM dasatinib, 1.0 µM bosutinib (SKI606) or 1.0 µM saracatinib as indicated. Cell lysates were subjected to SDSPAGE as described in Methods. Representative experiment.  

**E.** HCC1954 cells were treated for 24h with MEDICA as indicated. Cell lysates were prepared in SDS sample buffer with or without mercaptoethanol, subjected to SDSPAGE and analyzed by immunoblot for phospho-c-Src(Tyr416).  

**F.** HCC1954 cells were treated for 4-5h in triplicates with MEDICA as indicated. ROS production was determined by DCF fluorescence, H2O2 release into the culture medium was determined by HRP / Amplex Red reagent, and GSH/GSSG ratio was determined by luminescence based system as described in Methods. ROS production and H2O2 release of control cells are defined as 1.0. Mean ± SE. *- Significant as compared to the respective control (p< 0.05).  

**G.** *Upper frame:* HCC1954 cells were treated for 24h with MEDICA as indicated, and 10mM NAC added 1h prior to MEDICA.
lysates were subjected to SDS-PAGE as described in Methods. Representative experiment. Lower frame: HCC1954 cells were treated for 24h with 150μM MEDICA, and 10mM NAC added 1h prior to MEDICA. Cell lysates were subjected to SDS-PAGE without mercaptoethanol as described in Methods, and analyzed by immunoblot for c-Src. Representative experiment. H. HCC1954 cells were cultured for 24h in the presence of 200 μM MEDICA, 8 μgr/ml PEG or 100 units/ml PEG-SOD (Sigma) as indicated. Cells were trypsinized and subjected to clonogenic assay as described in Methods. Colonies exceeding 400 microns in size were counted. Clonogenity in the presence of PEG or PEG-SOD alone is defined as 1.0. Mean ± SE of 3 independent experiments. *- Significant as compared to the respective control (p< 0.05); #- Significant as compared to MEDICA/PEG (p< 0.05).
Figure 1

Panel A: Bar graph showing tumor mass (g) for Control, HFD, Keto, and MEDICA groups.

Panel B: H&E and BrdU stained images comparing Control (12) and Keto (4/12) groups.
Figure 2

A

Tumor mass (g)

B

Control

MEDICA

C

Control (12)

MEDICA (4/12)

D

Control

MEDICA (12/18)
Figure 4

A

Control (12)  MEDICA (4/12)

B

Control (12)  MEDICA (4/12)

C

MMP9 mRNA (au)

Control (12)  MEDICA (4/12)

D

Control (12)  MEDICA (4/12)
Figure 5

A) MET-1 cells
B) MMTV-PyMT cells

C) Western Blot
- IP: PyMT
- IB: c-Src
- MEDICA - 12h 24h

D) Western Blot
- IP: PyMT
- IB: c-Src

E) Graph
- Cell Growth (x10^4) vs. Time (days)
Long chain fatty acid analogs suppress breast tumorigenesis and progression

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