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Growth inhibitory and anti-metastatic activity of Epithelial cell adhesion molecule targeted three-way junctional Delta-5-Desaturase siRNA nanoparticle for breast cancer therapy

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Abstract

8-hydroxyoctanoic acid (8-HOA) produced through cyclooxygenase-2 (COX-2) catalyzed dihomo- γ -linolenic acid (DGLA) peroxidation in delta-5-desaturase inhibitory (D5D siRNA) condition showed an inhibitory effect on breast cancer cell proliferation and migration. Though, *in vivo* use of naked D5D siRNA was limited by off-target silencing and degradation by endonucleases. To overcome the limitation and deliver the D5D siRNA *in vivo*, we designed an epithelia cell adhesion molecule targeted three-way junctional nanoparticle having D5D siRNA. In this study, we have hypothesized that 3WJ-EpCAM-D5D siRNA will target and inhibit the D5D enzyme in cancer cells leading to peroxidation of supplemented DGLA to 8-HOA resulting in growth inhibitory effect in the orthotopic breast cancer model developed by injecting 4T1 cells. On analysis, we observed a significant reduction in tumor size and metastatic lung nodules in animals treated with a combination of 3WJ-EpCAM-D5D siRNA and DGLA through activating intrinsic apoptotic signaling pathway and by reducing endothelial-mesenchymal damage.

Graphical abstract

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Paradigm shift concept of COX-2 in breast cancer therapeutics

Dihomo- γ -Linolenic acid (DGLA) on metabolism produces Arachidonic Acid (AA) by the enzyme Delta-5-Desaturase (D5D). In cancer cells, produced AA further gets metabolized to precancerous two-series prostaglandins PGE₂ by Cyclooxygenase-2 (COX-2). The produced PGE₂ is known for its stimulatory effect on cancer growth and metastasis. However, inhibition of D5D by 3WJ-EpCAM-D5DsiRNA nanoparticle results in the accumulation of DGLA. The accumulated DGLA, by the action of overexpressed COX-2 in breast cancer, undergoes C-8 oxygenation to produce a free radical metabolite 8-Hydroxyoctanoic acid (8-HOA), which has anti-cancer and inhibitory effect on metastasis.

Keywords

3WJ-EpCAM-D5D siRNA; Apoptosis; Metastasis; Orthotopic breast cancer model; RNA nanoparticle

Introduction

As per the American Cancer Society, breast cancer is the most commonly diagnosed cancer in American women, with more than 275,000 expected cases in 2020 (1,2). There are many risk factors for breast cancer development and progression, out of which omega-6-polyunsaturated fatty acids (ω -6 PUFAs, a majority of the portion in western diet) is one of the factors responsible for breast cancer induction and progression (3-12). An upstream ω -6 PUFA, dihomo- γ -linolenic acid (DGLA), by the action of Delta-5-desaturase (D5D, an essential enzyme in the human body) produce arachidonic acid (AA). The generated AA in cancer cell metabolizes to procancerous two series prostaglandins (PGE₂) by overexpressed cyclooxygenase-2 (COX-2), resulting in breast cancer growth and metastasis through various pathways (8,11,18-21).

Recently, our lab has provided multiple evidences regarding anti-cancer activity in colon, pancreatic, and breast cancer through modifying ω -6 PUFAs metabolism by silencing D5D. In these studies, we have used D5D siRNA to inhibit DGLA metabolism. The accumulated DGLA underwent C-8 oxygenation by COX-2 to produce a free radical metabolite, 8-Hydroxyoctanoic acid (8-HOA), and exerted an inhibitory effect on cancer cell proliferation and migration (17-21). However, the utilized approach in the above studies have limitations, i.e., D5D siRNA stability, off-target silencing, and dissociation at an ultra-low concentration in the systemic environment (22). Hence, to achieve the therapeutic effect through this concept, it was necessary to identify the way to deliver D5D siRNA *in vivo*.

With the advancement in nanotechnology, nanosize material has gained global attention to gene delivery. Recently, some preliminary clinical trials of nanoparticle gene delivery showed a convincing effect (23). Despite being achieved a preliminary goal, the clinical development of nanoparticle is facing several inevitable challenges like encapsulation efficiency, stability and degradation in a physiological environment, endocytosis by target cells, endosomal escape, and toxicity (24). Recently, Dr. Shu's lab came up with the unique three-way junctional (3WJ) RNA nanoparticle delivery system, which can help to deliver the

required genetic material to the target site. The 3WJ nanoparticle can be self-assembled *via* bottom-up approach from three pieces of small RNA oligomers with high affinity. The resulting structure has high thermodynamic stability, significant long half-life compared to naked siRNA, metabolic durability, and resistance to degradation. In addition to these, due to the presence of three arms, 3WJ nanoparticle can be attached with the targeting moiety, detection moiety, and therapeutic moiety (22). Hence, to enhance the D5D siRNA delivery and to improve the therapeutic output, in the current study, we have used unique 3WJ nanoparticle harboring epithelial cell adhesion molecule (EpCAM, overexpressed on breast cancer cells) aptamer as a targeting module (25), Alexa 647 as an imaging moiety, and D5D siRNA as a therapeutic module to treat breast cancer in orthotopic breast cancer model induced by injecting 4T1 cells. Previously, we have used EpCAM targeted 3WJ nanoparticle-containing D5D siRNA (3WJ-EpCAM-D5D siRNA) to redirect DGLA metabolism to achieve an anti-cancer activity in colon cancer model by producing an anti-cancer free radical metabolite 8-HOA (26). However, the paradigm shift approach of COX-2 to alter the DGLA metabolism by 3WJ-EpCAM-D5D siRNA in breast cancer has not yet been explored. Here, we have hypothesized that systemic injection of 3WJ-EpCAM-D5D siRNA will target and deliver the D5D siRNA to the breast cancer cells, resulting in the inhibition of D5D expression. The decrease of D5D will inhibit the metabolism of administered DGLA to AA resulting in less production of PGE₂, and COX-2 will peroxidize the accumulated DGLA to an anti-cancer metabolite 8-HOA. The generated 8-HOA will cause apoptosis in cancer cells and inhibit the metastasis of cancer cells.

Methods:

Cell lines:

4T1, high COX-2 expressing mouse breast cancer cell line, and MCF-12a, human epithelial non-cancerous cell line, were purchased from ATCC. Both cells were grown as per the standard culture protocol mentioned at ATCC.

Synthesis of 3WJ RNA nanoparticles:

In this study, we have used four types of nanoparticles, a nanoparticle with targeting module (3WJ-EpCAM), the second type has imaging module (3WJ-Alexa 647), the third type has targeting and imaging module (3WJ-EpCAM-Alexa 647), and the nanoparticle with targeting and therapeutic module (3WJ-EpCAM-D5D siRNA). All the nanoparticles were made as per the procedure mentioned in Xu et al. (26). Additional details are provided in the supplemental materials.

Cell binding of 3WJ-EpCAM-Alexa 647 nanoparticles:

Approximately 3×10^5 4T1 cells were trypsinized (Hyclone Laboratories, IL, USA) and washed. After centrifugation, 4T1 cells were resuspended in PBS or 100nM 3WJ-EpCAM-Alexa 647 nanoparticle (ExonanoRNA, Ohio, USA) and incubated at 37°C for 2 hours. After the incubation, cells were washed and resuspended in PBS, and subjected to flow cytometry analysis (BD Accuri C6 Flow Cytometer, NJ, USA) for Alexa 647 intensity determination (26).

Cell internalization of 3WJ-EpCAM-Alexa 647 nanoparticles:

About $1.5\text{--}2.0 \times 10^3$ 4T1 cells were seeded into 8 well μ -Slide (ibidi, Fitchburg, WI, USA). Cells were treated with 200nM 3WJ-EpCAM-Alexa 647 nanoparticle for 2 and 4 hours. After the incubation, the cells were washed and fixed. Next, the cells were incubated with Phalloidin 488 (Abcam) and washed. Then nucleus was stained by Fluoro-Gel II with DAPI (Electron Microscopy Sciences, PA, USA). Then the stained cells were observed under a confocal microscope (LSM900 with Airyscan 2, Carl Zeiss Microscopy, NY, USA) (26).

Wound healing assay:

Wound healing assay was carried out as mentioned before in our previous studies (17-20). Detailed description of the procedure is provided in the supplemental materials.

Transwell assay:

Transwell assay was performed as per the procedure mentioned in our previous study (17). Please refer to supplemental materials for the complete description.

***In vivo* biodistribution of nanoparticles:**

Five weeks old female homozygous nude mice (NU/J) were procured from The Jackson Laboratory (Sacramento, USA) for the animal study. The protocol for animal study was approved by the Institutional Animal Care and Use Committees (IACUC) at North Dakota State University. After two weeks of acclimatization, mice were injected 0.25×10^6 4T1 cells on the fourth mammary fat pad to induce breast cancer and allowed 20 days for the tumor to grow. Once the tumor reached the required size, mice in different groups were injected 20 μ M 3WJ-EpCAM nanoparticle, 20 μ M 3WJ-EpCAM-Alexa 647, and 20 μ M 3WJ-Alexa 647 nanoparticle through the tail vein. After eight hours, animals were euthanized, tumors and vital organs (heart, lung, liver, kidney, and spleen) were harvested. The *in vivo* biodistribution of 3WJ-nanoparticle harboring Alexa 647 was determined by the In Vivo Imaging System (IVIS) Spectrum station (26).

Orthotopic breast cancer model to evaluate the effect of 3WJ-EpCAM-D5D siRNA nanoparticle:

After two weeks of acclimatization, 0.25×10^6 4T1 cells were injected in the fourth mammary fat pad of the mice and allowed the tumor to grow (27,28). After ten days of the incubation period, the mice were randomly assigned into four different treatment groups (n=4); Vehicle treated group, DGLA (Cayman chemicals, MI, USA) group (5mg/mouse, *p.o.*, every day), 3WJ-EpCAM-D5D siRNA group (20 μ M through *i.v.* route, twice a week), and combination of DGLA + 3WJ-EpCAM-D5D siRNA group. During the treatment period, the animal weight and tumor size was measured every three days by a digital Vernier caliper. After the treatment period, tumors from different groups of animals and vital organs were collected and processed.

Quantification of 8-HOA via GC/MS:

The tumor tissues were processed as mentioned before to determine 8-HOA (17-19) and detailed description is mentioned in supplemental materials.

Quantification of DGLA and AA by LC/MS:

The DGLA and AA in the tumor were determined as per the procedure described in previous studies (17-19) and complete description is mentioned in supplemental materials.

HDAC activity determination:

Protein extracted from the animal tumors was used to determine the Histone Deacetylase (HDAC) activity using commercial kit (The HDAC activity colorimetric assay kit, BioVision, CA, USA). All the steps were performed as per the instructions provided in the kit.

Western blot analysis:

The whole cell lysate from 4T1, MCF-12a, and tumor was prepared by using RIPA buffer premixed with protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, MA, USA). Lysate was resolved on 10% SDS-PAGE using Bio-Rad miniprotein II gels and electroblotted to polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific) through Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, CA, USA). The membrane was blocked and incubated with various primary (D5D, E-Cadherin, MMP-9 (Abcam, MA, USA); Cytochrome C, Bcl-2, Procaspase-9, γ -H2Ax, Cleaved PARP, MMP-2, β -actin (Cell signaling, MA, USA); Vimentin, EpCAM (Santacruz Biotechnology, TX, USA)) and secondary (IRDye from Li-Cor, NE, USA) antibodies. A Li-Cor Odyssey XL System was used to capture the protein signals, and densitometry analysis was performed by using Image Studio v.5.2 software (17-19).

Immunofluorescence analysis:

To determine the D5D knocking down efficiency of 3WJ-EpCAM-D5D siRNA, approximately $1.5-2.0 \times 10^3$ 4T1 cells were seeded into each well of μ -Slide 8 Well (ibidi). The cells were then incubated with 100nM 3WJ-EpCAM-D5D siRNA for 48hours. After treatment, cells were washed, fixed, and blocked. Followed by blocking, the 4T1 cells were incubated with the D5D primary antibody (Abcam) and Alexa 647 (Abcam) secondary antibodies. The cells were then washed and stained with Phalloidin-iFluor 488 Reagent. Nucleus was stained by DAPI. The fluorescence images of 4T1 cells were captured by LSM900 confocal microscopy with Airyscan 2 (Carl Zeiss Microscopy).

The expression of D5D, Cleaved PARP, Ki-67, Vimentin, MMP-2, and E-Cadherin in tumor tissue were processed as described previously (26). The tumor sections on slides were incubated with various primary antibodies and then with fluorochrome-conjugated secondary antibodies. DAPI was used to stain the cell nuclei. The images were taken by using LSM900 confocal microscopy with Airyscan 2 (Carl Zeiss Microscopy) by using confocal mode.

Hematoxylin and eosin (H&E) staining:

Organs from animals treated with different treatments were harvested and sent to Advanced Imaging & Microscopy Laboratory at North Dakota State University for H&E staining. The

images of tissue samples were obtained from an inverted microscope (Lecia Microsystems Model DMI8) in the bright field mode.

Statistics:

Statistical analysis was conducted using Prism 5 GraphPad. *In vitro* and *in vivo* measurement data were presented as the means±standard error of the mean (SEM). Multiple comparisons among groups were evaluated using a one-way ANOVA followed by Bonferroni test. Differences with minimum of $p<0.05$ were indicated as statistically significant. All the experiments were repeated at least three times.

Results

3WJ nanoparticle synthesis and structure confirmation

3WJ motif derived from pRNA molecule used as a skeleton to attach different molecules such as imaging Alexa 647, EpCAM targeting aptamer, and therapeutic D5D siRNA based on the use during the study (Figure 1A). The image from native PAGE run confirmed successful assembly of the nanoparticles (Figure 1B).

In vitro effect of 3WJ nanoparticles on 4T1 cells

To determine cell targeting efficiency of 3WJ nanoparticle harboring EpCAM, we incubated 3WJ-EpCAM-Alexa 647 nanoparticles with 4T1 (EpCAM positive) cells. Flow cytometry analysis demonstrated the shift in peak (orange) in the group of cells treated with 3WJ-EpCAM-Alexa 647 nanoparticle compared to the Vehicle-treated group of cells (blue), indicating the desirable targeting efficiency of 3WJ-EpCAM nanoparticle (Figure 1C). In addition to this, confocal microscopy images showed that the 3WJ-EpCAM-Alexa 647 nanoparticles were able to enter the cells efficiently at 4hours (Figure 1D). We also analyzed the nanoparticle internalization at 2hours, but we did not observe any nanoparticle internalization (Supplementary Figure 1). We believe that at 2hours, the nanoparticles might have bind to the EpCAM on cell membrane, but only less amount of them have entered the cells. D5D is a widely expressed enzyme in the human body. To analyze the D5D knocking down efficiency, we treated the 4T1 cells with 10nM 3WJ-EpCAM-D5D siRNA nanoparticle, and performed Western and microscopy analysis. In Western analysis, we provided 6hours treatment of 3WJ-EpCAM-D5D siRNA and recovered the protein at 12 and 24hours. On analysis, we observed a significant reduction of D5D protein expression in 4T1 cells after 12 and 24hours of treatment (Figure 1E, $p<0.001$ and $p<0.05$) and confocal microscopy analysis also displayed significant reduction (~53%) in D5D protein expression after treatment with 3WJ-EpCAM-D5D siRNA nanoparticle treatment in 4T1 cells (Figure 1F, $p<0.05$).

In vivo tumor targeting and tumor-suppressing effect of 3WJ nanoparticles

To determine the *in vivo* effect of 3WJ-nanoparticles, different types of nanoparticles, 3WJ-EpCAM, 3WJ-Alexa 647, 3WJ-EpCAM-Alexa 647 and 3WJ-EpCAM-D5D siRNA were used in two separate studies. Before determining *in vivo* targeting and therapeutic effects, orthotopic breast cancer was established by injecting 4T1 cells into the 4th mammary fat pad of Nu/J mice and quarantined for a different time (Figure 2A and 2B).

In the first set of studies, to determine biodistribution of 3WJ-EpCAM-Alexa 647 and 3WJ-Alexa 647 nanoparticles, animals were housed until the desired tumor size was achieved. After 20 days of cell implantation, saline, 3WJ-Alexa 647, and 3WJ-EpCAM-Alexa 647 nanoparticles were injected through *i.v.* route. After eight hours of nanoparticle injection, mice were euthanized, and different organs and tumors were harvested. On the distribution analysis, a higher amount of 3WJ-EpCAM-Alexa 647 accumulation was observed in the cancer tissue (indicated by higher fluorescence) and negligible amount in the liver. In contrast, animals receiving 3WJ-Alexa 647 nanoparticles showed distribution in majority of the harvested organs (liver, lung, spleen, and kidneys). Animals receiving saline as control did not display any fluorescence (Figure 2C). This could be due to observed overexpression of EpCAM in the breast cancer tissue (Supplementary Figure 2), which might have facilitated the targeted delivery of nanoparticles to the tumor.

In the second study, to determine the growth-inhibitory efficacy of 3WJ-EpCAM-D5D siRNA, as shown in Figure 2B, after ten days of 4T1 cell injection, animals were randomized into four different groups; Vehicle, DGLA (5mg), 3WJ-EpCAM-D5D siRNA (20 μ M), and combination of DGLA and 3WJ-EpCAM-D5D siRNA. After one month, tumor size analysis data indicated that the combination treatment of DGLA and 3WJ-EpCAM-D5D siRNA could lead to a significant reduction in tumor size, which was also evident by tumor pictures (Figure 2D and 2E, $p<0.01$). As per the research strategy, inhibition of D5D by 3WJ-EpCAM-D5D siRNA leads to inhibition of the D5D enzyme, which results in the diversion of DGLA metabolism to 8-HOA production. Immunofluorescence analysis and Western analysis of tumor tissues harvested from different groups of animals suggested that treatment with 3WJ-EpCAM-D5D siRNA alone or in combination with DGLA was able to significantly knock down the D5D protein level in breast cancer (Figure 2F, $p<0.05$ and 3F, $p<0.01$).

3WJ-EpCAM-D5D siRNA redirected the DGLA peroxidation and induced apoptosis *in vivo*

In physiological conditions, the essential ω -6 polyunsaturated fatty acid DGLA metabolizes to AA due to the D5D activity. The inhibition of D5D enzyme imbalances DGLA and AA level in the cell resulting in accumulation of DGLA. The accumulated DGLA eventually peroxidized to anti-cancer metabolite 8-HOA by overexpressed COX-2 in cancer cells (17-20). The LC-MS analysis suggested similar findings that the D5D inhibition by 3WJ-EpCAM-D5D siRNA resulted in the significant downregulation of AA and upregulation of the DGLA/AA ratio in breast tumor tissues (Figure 3B and 3C, $p<0.01$). Through GC-MS analysis, we detected significant increases in 8-HOA (0.704 ± 0.05) production in tumor tissue when animals were treated with the combination of DGLA and 3WJ-EpCAM-D5D siRNA. Animals receiving Vehicle, DGLA, or 3WJ-EpCAM-D5D siRNA treatment showed below threshold ($>0.5\mu$ M, the minimum level required to produce a therapeutic effect) 8-HOA level (0.25 ± 0.07 , 0.125 ± 0.051 , and 0.256 ± 0.23 , respectively) (Figure 3A, $p<0.05$).

To identify possible molecular mechanism responsible for cancer growth inhibitory activity, we performed immunohistochemistry and Western. These studies highlighted that the breast cancer growth inhibitory activity of DGLA and 3WJ-EpCAM-D5D siRNA combination was through the significant upregulation of apoptosis marker Cleaved poly (ADP-Ribose)

polymerase (C. PARP) (Figure 3E, $p<0.001$ and 3F, $p<0.001$). The concomitant administration of DGLA and 3WJ-EpCAM-D5D siRNA was also led to the downregulation of proliferation marker Ki-67 in tumor tissue (Figure 3E, $p<0.01$). On analyzing the relative protein expression related to the intrinsic apoptotic pathway, we acknowledged a significant ($p<0.01$) increase in Cytochrome C (Cyt. C) level and significant ($p<0.05$) decrease in procaspase-9 level associated with simultaneous treatment of DGLA and 3WJ-EpCAM-D5D siRNA in breast tumor tissue (Figure 3F). Additionally, we also witnessed that concurrent treatment of 3WJ-EpCAM-D5D siRNA and DGLA caused a significant ($p<0.001$) upregulation of DNA damage marker $\gamma\text{H}_2\text{Ax}$ and downregulation of anti-apoptotic protein Bcl-2 (Figure 3F). HDAC activity analysis by HDAC activation kit deciphered that treatment with 3WJ-EpCAM-D5D siRNA and DGLA could suppress HDAC activity in breast tumor tissues compared to animals receiving Vehicle, DGLA, or 3WJ-EpCAM-D5D siRNA alone as a treatment (Figure 3D, $p<0.01$).

Anti-metastatic effect of 3WJ-EpCAM-D5D siRNA nanoparticle and DGLA

To decipher whether the 3WJ-EpCAM-D5D siRNA in combination with DGLA have any effect on cancer cell migration, we performed wound healing assay and transwell assay. Wound healing assay showed significant ($p<0.001$) reduction in cell migration on combination treatment with DGLA and 3WJ-EpCAM-D5D siRNA group, whereas individual treatment with DGLA or 3WJ-EpCAM-D5D siRNA did not show any significant effect on cell migration (Supplementary Figure 3). Similarly, we observed a significant ($p<0.01$) reduction in cell migration by combination treatment across the membrane from transwell (Supplementary Figure 4). This captivated us to analyze the *in vivo* effect of 3WJ-EpCAM-D5D siRNA against tumor metastasis.

Orthotopic breast cancer model established by inoculating 4T1 cells in the 4th mammary fat pad likely to produce metastatic lung nodules within 10-15 days of cell injection. At the end of the treatment period, lungs from different groups of animals were harvested and observed for metastatic lung nodules. By counting the number of nodules on the lungs, we found that the metastasized lung nodules on mice treated with the combination of DGLA and 3WJ-EpCAM-D5D siRNA were less (two nodules) than the Vehicle (eight nodules), DGLA (five nodules), or 3WJ-EpCAM-D5D siRNA treated animals (six nodules, Figure 4A). H&E staining of lung sections confirmed similar findings as morphological findings (highlighted by yellow dotted lines, Figure 4B).

The Immunofluorescence analysis depicted decreased intensity of Matrix Metalloproteinases-2 (MMP-2) and Vimentin in the tumors harvested from animals treated with simultaneous 3WJ-EpCAM-D5D siRNA and DGLA (Figure 4D, $p<0.01$). Similarly, on analyzing the relative protein level through Western analysis, the significant downregulation of MMP-2, MMP-9, and Vimentin was observed in tumor tissues from animals treated with 3WJ-EpCAM-D5D siRNA and DGLA (Figure 4C, $p<0.01$). However, the administration of DGLA or 3WJ-EpCAM-D5D siRNA alone barely influenced the protein expression of Vimentin, MMP-9, and MMP-2 in tumor tissues. Besides, significant upregulation was observed in epithelial cadherin (E-cadherin) fluorescence intensity in mice receiving concomitant treatment with DGLA and 3WJ-EpCAM-D5D siRNA (Figure 4C and 4D,

$p<0.001$). In contrast, no significant change of E-cadherin level was found in the mice receiving DGLA or 3WJ-EpCAM-D5D siRNA alone (Figure 4C and 4D).

Discussion

There are several risk factors for breast cancer development, including consumption of ω -6 PUFAs, like DGLA, which is present in the majority of the western diet (4-6). The D5D enzyme in cells metabolizes DGLA to AA, which sequentially peroxidized to PGE₂ by COX-2 (13-16). Hence, by considering COX-2 as a risk, many research groups have analyzed the protective role of COX-2 inhibitors in cancer management at *in vitro* and murine study level. However, COX-2 inhibitors have failed to improve the patients' survival in several clinical studies (29-31). Notably, the use of the COX-2 inhibitor was associated with life-threatening cardiac adverse events (32). Recently, our lab provided evidence regarding manipulated metabolism of DGLA by knocking down D5D, which lead to oxygenation of DGLA to an anticancer metabolite, 8-HOA (17-21,26). However, siRNA use is linked with disadvantages, such as off-target silencing and dissociation of genetic material in the systemic circulation (22). Hence, to overcome the limitations and improve the delivery efficiency of D5D siRNA to the cancer cell to achieve anti-cancer activity, we employed thermodynamically and kinetically stable 3WJ-nanoparticle technology having EpCAM aptamer as a targeting arm, D5D siRNA as a therapeutic arm, and Alexa 647 as an imaging arm. As per our previously published report, the 3WJ nanoparticles were approximately 5.8 ± 1.1 nm. To visualize the branched global structure of nanoparticles, we have used atomic force microscopy (AFM). Since the size of the nanoparticle was out of the detection range of AFM probe, we extended the individual arms of the nanoparticles with 60bp of dsRNA extension, as established in our recent work (26).

As indicated before by other groups, breast cancer cells overexpress EpCAM from 100-1000 fold compared to healthy breast tissue (25,33). This concurs with our current findings on high expression of EpCAM in breast cancer cells (4T1), where low or no expression in non-cancerous breast tissue cells (MCF-12a). In this study, 3WJ-pRNA nanoparticle having EpCAM aptamer as a targeting module was successfully able to bind and enter the breast cancer cells. Once entering the breast cancer cells, the 3WJ-EpCAM-D5D siRNA significantly inhibited the D5D expression, as indicated by reduced D5D expression in 4T1 cells at 12hours from 3WJ-EpCAM-D5D siRNA treatment. Interestingly, we observed siRNA inhibition of D5D at 24hours seems less than 12hours, this could be due to the doubling time (13.6 ± 1.5 hours) of 4T1 cells, as noted in elsewhere (34). In addition to this, the *In vivo* imaging analysis also concluded that the 3WJ-EpCAM nanoparticle could target and deliver a significant amount of the D5D siRNA to the tumor in animals. However, unlike the low *in vivo* efficiency of naked siRNA, immunohistochemistry analysis concluded that 3WJ-EpCAM-D5D siRNA±DGLA could significantly inhibit the D5D enzyme in breast tumor tissues. Due to the successfully knocking down of D5D, the COX-2 catalyzed DGLA peroxidation was redirected from the production of AA to 8-HOA, which could play a significant role in the inhibition of breast tumor growth in nude mice.

In line with the previously published results by using D5D siRNA (*in vitro*) and D5D shRNA (*in vivo*), DGLA co-administration with 3WJ-EpCAM-D5D siRNA was able to

produce a significant amount of 8-HOA in cancer cells/tumors (17-19,21,26). Additionally, combination treatment was also able to furnish high DGLA/AA ratio, which is an indicator of D5D inhibition. Relevant to our hypothesis, *in vivo* inhibition of D5D enzyme obstructs the DGLA metabolism to AA resulting in less production of AA and the accumulated DGLA undergoes C-8 oxygenation to produce 8-HOA is achieved by using 3WJ-EpCAM-D5D siRNA. Therapeutic pathway analysis showed that the DGLA and 3WJ-EpCAM-D5D siRNA combination was able to activate the intrinsic apoptotic pathway as displayed by high expression of Cytochrome C, apoptotic marker (C.PARP), and reduced expression of procaspase-9 and Bcl-2. Not only activating apoptosis, the 3WJ-EpCAM-D5D siRNA also could downregulate the proliferation of breast cancer cells as indicated by downregulation in proliferation marker Ki-67. In breast cancer progression, increased HDAC activity could decrease the transcription of tumor suppressor gene (such as p21) and unchecked the cell growth lead to more proliferation but less apoptosis in cancer cells (35). Interestingly, we also noted that endogenously produced 8-HOA, from 3WJ-EpCAM-D5D siRNA induced DGLA peroxidation, was significantly able to reduce the HDAC activity and increase the DNA damage as indicated by high expression of double-strand damage marker $\gamma\text{H}_2\text{Ax}$. As the potential HDAC inhibitor, generated 8-HOA may loosen the compact DNA by hyperacetylation of histone and result in double-strand breaks in DNA and, ultimately cancer cell death (36).

In 4T1 induced breast cancer orthotopic model, breast cancer cells leave the solid tumor site and migrate to distant organs such as lung, and grow to form a metastatic lung nodule. (27). During breast cancer invasion and metastasis, cancer cells overexpresses zinc-dependent proteases such as MMP-2 and MMP-9 (37). The secreted MMP-2 and MMP-9 from tumor cells degrade type IV collagen, which is an essential part of the basement membrane. High expression of MMP-2 and MMP-9 leads to migration of breast cancer cells from the damaged membrane into the systemic circulation, leading to tumor invasion and metastasis (37-39). Our *in vitro* wound healing and transwell assay, and the number of lung metastatic nodules from *in vivo* study showed reduced breast cancer cell migration on treatment with DGLA and 3WJ-EpCAM-D5D siRNA combination. On dissecting the molecular pathway, we identified that the concomitant DGLA and 3WJ-EpCAM-D5D siRNA administration was significantly able to reduce MMP-2 and MMP-9. Vimentin is a principle cytoskeletal protein constituent of the intermediate filament, which is required for metastasis (40). Vimentin was downregulated with the simultaneous treatment of DGLA and 3WJ-EpCAM-D5D siRNA. In addition to this, we also observed upregulated E-cadherin (single-span transmembrane glycoprotein required to build epithelial adherens junction, disruption of which leads to metastasis (41)) with the concomitant administration of DGLA and 3WJ-EpCAM-D5D siRNA. Reduced expression of MMP-2, MMP-9, Vimentin, and upregulated E-cadherin from DGLA and 3WJ-EpCAM-D5D siRNA treatment is responsible for reduced metastasis, which is evident from morphological and H&E analysis of lungs showing significantly less number of metastatic lung nodules in the animals.

In addition to this, we also measured animal body weight during the treatment, and we did not observe any significant change in body weight (data not shown). The H&E staining of organs (liver, heart, spleen, and kidney) showed no sign of abnormality in any of the treatment groups (data not provided). These two findings lead us to believe that pRNA

delivery system, DGLA, and combination of 3WJ-EpCAM-D5D siRNA and DGLA does not have any toxic effect. The toxicity status of the delivery system is also supported by the previous study in which 3WJ-EpCAM-D5D siRNA harbored with EpCAM aptamer, and anti-micro RNA 21 has high biocompatibility and less toxic effect in the treatment of triple-negative breast cancer (42).

To summarize, 3WJ-EpCAM-D5D siRNA can target and deliver the D5D siRNA to the breast cancer tissue and inhibit the D5D expression. This effect hinders the DGLA metabolism to AA. On the contrary, accumulated DGLA undergo peroxidation to 8-HOA by overexpressed COX-2, which produce growth inhibitory and anti-metastatic effects in breast cancer. Besides, the utilization of the pRNA 3WJ delivery system provided the targeted approach, which improved the therapeutic outcome and reduced off-targeted silencing effects. Hence, by delivering the D5D siRNA to inhibit the D5D and exploiting the use of overexpressed COX-2 in cancer therapy is an effective approach for breast cancer management.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ω-6 PUFAs	Omega-6-polyunsaturated fatty acids
DGLA	Dihomo-gamma-linolenic acid
AA	Arachidonic acid
D5D	Delta-5-Desaturase
PGE₂	Prostaglandin E ₂
COX-2	Cyclooxygenase-2
8-HOA	8-Hydroxy octanoic acid
3WJ	Three way junctional
EpCAM	Epithelial cell adhesion molecule
NP	Nanoparticle

PFB-bromide	Pentafluorobenzyl-bromide
SPE	Solid phase electrophoresis
PAGE	Poly acrylamide gel electrophoresis
IACUC	Institutional Animal Care and Use Committees
IVIS	In Vivo Imaging System
SEM	Standard error of the mean
HDAC	Histone deacetylase
PVDF	Polyvinylidene difluoride
C.PARP	Cleaved poly (ADP-Ribose) polymerase
Cyt. C	Cytochrome C
H&E	Hematoxylin and eosin

References

1. Facts C ACS 2020 Facts and Figures. 2020 ([Cancer.org](https://www.cancer.org)).
2. DeSantis CE, Ma J, Gaudet MM, Newman LA, Miller KD, Goding Sauer A, et al. Breast cancer statistics, 2019. *CA Cancer J Clin*. 2019;69(6):438–51. [PubMed: 31577379]
3. Huerta-Yépez S, Tirado-Rodriguez AB, Hankinson O. Role of diets rich in omega- 3 and omega-6 in the development of cancer. *Bol Med Hosp Infant Mex*. 2016;73(6):446–56. [PubMed: 29421289]
4. Bartsch H, Nair J, Owen RW. Dietary polyunsaturated fatty acids and cancers of the breast and colorectum: Emerging evidence for their role as risk modifiers. *Carcinogenesis*. 1999;20(12):2209–18. [PubMed: 10590211]
5. Hidaka BH, Li S, Harvey KE, Carlson SE, Sullivan DK, Kimler BF, et al. Omega-3 and omega-6 fatty acids in blood and breast tissue of high-risk women and association with atypical cytomorphology. *Cancer Prev Res*. 2015;8(5):359–64.
6. Zanoaga O, Jurj A, Raduly L, Cojocneanu-Petric R, Fuentes-Mattei E, Wu O, et al. Implications of dietary ω -3 and ω -6 polyunsaturated fatty acids in breast cancer. *Exp Ther Med*. 2018; 15(2): 1167–76. [PubMed: 29434704]
7. Thiebaut ACM, Chajes V, Gerber M, Boutron-Ruault M-C, Joulin V, Lenoir G, et al. Dietary intakes of omega-6 and omega-3 polyunsaturated fatty acids and the risk of breast cancer. *Int J cancer*. 2009;124(4):924–31. [PubMed: 19035453]
8. Godley PA. Essential fatty acid consumption and risk of breast cancer. *Breast Cancer Res Treat*. 1995;35(1):91–5. [PubMed: 7612909]
9. Chajès V, Torres-Mejía G, Biessy C, Ortega-Olvera C, Angeles-Llerenas A, Ferrari P, et al. ω -3 and ω -6 polyunsaturated fatty acid intakes and the risk of breast cancer in Mexican women: Impact of obesity status. *Cancer Epidemiol Biomarkers Prev*. 2012;21(2):319–26. [PubMed: 22194528]
10. Khodarahmi M, Azadbakht L. The association between different kinds of fat intake and breast cancer risk in women. *Int J Prev Med*. 2014;5(1):6–15. [PubMed: 24554986]
11. MacLennan M, Ma DWL. Role of dietary fatty acids in mammary gland development and breast cancer. *Breast Cancer Res*. 2010; 12(5):211. [PubMed: 21067551]
12. Fabian CJ, Kimler BF, Hursting SD. Omega-3 fatty acids for breast cancer prevention and survivorship. *Breast Cancer Res*. 2015;17(1):62. [PubMed: 25936773]
13. Gu Z, Shan K, Chen H, Chen YQ. n-3 Polyunsaturated Fatty Acids and Their Role in Cancer Chemoprevention. *Curr Pharmacol Reports*. 2015;1(5):283–94.

14. Bagga D, Wang L, Farias-Eisner R, Glaspy JA, Reddy ST. Differential effects of prostaglandin derived from ω -6 and ω -3 polyunsaturated fatty acids on COX-2 expression and IL-6 secretion. *Proc Natl Acad Sci USA*. 2003; 100(4): 1751–6. [PubMed: 12578976]
15. Azrad M, Turgeon C, Demark-Wahnefried W. Current evidence linking polyunsaturated fatty acids with cancer risk and progression. *Front Oncol*. 2013;3:1–12. [PubMed: 23373009]
16. Yang P, Jiang Y, Fischer SM. Prostaglandin E3 metabolism and cancer. *Cancer Lett*. 2014;348(1–2):1–11. [PubMed: 24657656]
17. Xu Y, Yang X, Wang T, Yang L, He Y, Miskimins K, et al. Knockdown delta-5-desaturase in breast cancer cells that overexpress COX-2 results in inhibition of growth , migration and invasion via a dihomo- γ -linolenic acid peroxidation dependent mechanism. *BMC Cancer*. 2018; 18(330): 1–15. [PubMed: 29291726]
18. Xu Y, Yang X, Zhao P, Yang Z, Yan C, Guo B, et al. Knockdown of delta-5-desaturase promotes the anti-cancer activity of dihomo-gamma-linolenic acid and enhances the efficacy of chemotherapy in colon cancer cells expressing COX-2. *Free Radic Biol Med*. 2016;96:67–77. [PubMed: 27101738]
19. Xu Y, Qi J, Yang X, Wu E, Qian SY. Free radical derivatives formed from cyclooxygenase-catalyzed dihomo-gamma-linolenic acid peroxidation can attenuate colon cancer cell growth and enhance 5-fluorouracil's cytotoxicity. *Redox Biol*. 2014;2:610–8. [PubMed: 25114837]
20. Yang X, Xu Y, Brooks A, Guo B, Miskimins KW, Qian SY. Knockdown delta-5-desaturase promotes the formation of a novel free radical byproduct from COX-catalyzed omega-6 peroxidation to induce apoptosis and sensitize pancreatic cancer cells to chemotherapy drugs. *Free Radio Biol Med*. 2016;97:342–50.
21. Xu Y, Qian SY. Anti-cancer activities of omega-6 polyunsaturated fatty acids. *Biomed J*. 2014;37(3):112–9. [PubMed: 24923568]
22. Gavrillov K, Saltzman WM. Therapeutic siRNA: Principles, challenges, and strategies. *Yale J Biol Med*. 2012;85(2): 187–200. [PubMed: 22737048]
23. Hanna J, Hossain GS, Kocerha J. The potential for microRNA therapeutics and clinical research. *Front Genet*. 2019; 10. [PubMed: 30815010]
24. Chen J, Guo Z, Tian H, Chen X. Production and clinical development of nanoparticles for gene delivery. *Mol Ther - Methods Clin Dev*. 2016;3:16023. [PubMed: 27088105]
25. Osta WA, Chen Y, Mikhitarian K, Mitas M, Salem M, Hannun YA, et al. EpCAM is overexpressed in breast cancer and is a potential target for breast cancer gene therapy. *Cancer Res*. 2004;64(16):5818–24. [PubMed: 15313925]
26. Xu Y, Pang L, Wang H, Xu C, Shah H, Guo P, et al. Specific delivery of delta-5-desaturase siRNA via RNA nanoparticles supplemented with dihomo- γ -linolenic acid for colon cancer suppression. *Redox Biol*. 2019;21.
27. Fantozzi A, Christofori G. Mouse models of breast cancer metastasis. *Breast Cancer Res*. 2006;8(4).
28. Sun X, Ma X, Li Q, Yang Y, Xu X, Sun J, et al. Anti-cancer effects of fisetin on mammary carcinoma cells via regulation of the PI3K/Akt/mTOR pathway: In vitro and in vivo studies. *Int J Mol Med*. 2018;42(2):811–20. [PubMed: 29749427]
29. Harris RE, Chlebowski RT, Jackson RD, Vogel VG. Breast cancer and nonsteroidal anti-inflammatory drugs; prospective results from the women's health initiative. *Breast Dis*. 2004; 15(2): 134–5.
30. Johnson TW, Anderson KE, Lazovich D, Folsom AR. Association of aspirin and nonsteroidal anti-inflammatory drug use with breast cancer. *Cancer Epidemiol Biomarkers Prev*. 2002; 11 (12): 1586–91. [PubMed: 12496048]
31. Dang CT, Dannenberg AJ, Subbaramaiah K, Dickler MN, Moasser MM, Seidman AD, et al. Phase II study of celecoxib and trastuzumab in metastatic breast cancer patients who have progressed after prior trastuzumab-based treatments. *Clin Cancer Res*. 2004; 10(12 Pt 1):4062–7. [PubMed: 15217939]
32. Solomon SD, McMurray JJ V, Pfeffer MA, Wittes J, Fowler R, Finn P, et al. Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. *N Engl J Med*. 2005;352(11): 1071–80. [PubMed: 15713944]

33. Jenkins S V, Nima ZA, Vang KB, Kannarpady G, Nedosekin DA, Zharov VP, et al. Triple-negative breast cancer targeting and killing by EpCAM-directed, plasmonically active nanodrug systems. *npj Precis Oncol.* 2017; 1(1): 1–9. [PubMed: 29872691]
34. Simões R V, Serganova IS, Kruchevsky N, Leftin A, Shestov AA, Thaler HT, et al. Metabolic Plasticity of Metastatic Breast Cancer Cells: Adaptation to Changes in the Microenvironment. *Neoplasia.* 2015;17(8):671–84. [PubMed: 26408259]
35. Ropero S, Esteller M. The role of histone deacetylases (HDACs) in human cancer. *Mol Oncol.* 2007; 1(1): 19–25. [PubMed: 19383284]
36. Zhang Y, Adachi M, Zou H, Hareyama M, Imai K, Shinomura Y. Histone deacetylase inhibitors enhance phosphorylation of histone H2AX after ionizing radiation. *Int J Radiat Oncol Biol Phys.* 2006;65(3):859–66. [PubMed: 16751067]
37. Li H, Qiu Z, Li F, Wang C. The relationship between MMP-2 and MMP-9 expression levels with breast cancer incidence and prognosis. *Oncol Lett.* 2017; 14(5):5865–70. [PubMed: 29113219]
38. Kleiner DE, Stetler-Stevenson WG. Matrix metalloproteinases and metastasis. *Cancer Chemother Pharmacol.* 1999;43 Suppl:S42–51. [PubMed: 10357558]
39. El Deryugina, Quigley JP. Matrix metalloproteinases and tumor metastasis. *Cancer Metastasis Rev.* 2006;25(1):9–34. [PubMed: 16680569]
40. Wang W, Yi M, Zhang R, Li J, Chen S, Cai J, et al. Vimentin is a crucial target for anti-metastasis therapy of nasopharyngeal carcinoma. *Mol Cell Biochem.* 2018;438(1–2):47–57. [PubMed: 28744809]
41. Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer Res.* 2008;68(10):3645–54. [PubMed: 18483246]
42. Shu D, Li H, Shu Y, Xiong G, Carson WE, Haque F, et al. Systemic Delivery of Anti-miRNA for Suppression of Triple Negative Breast Cancer Utilizing RNA Nanotechnology. *ACS Nano.* 2015;9(10):9731–40. [PubMed: 26387848]

Figure 1: *In vitro* effect of 3WJ-EpCAM-D5D siRNA / 3WJ-EpCAM-Alexa 647 nanoparticles. **A)** RNA structure of 3WJ nanoparticle. **B)** Native PAGE showing a highly efficient assembly of the RNA nanoparticle. **C)** Cell targeting efficacy analysis of 3WJ-EpCAM-Alexa 647 nanoparticle by flow cytometry. In control, cells were treated with PBS (blue peak), and in treatment, cells were treated with 3WJ-EpCAM-D5D siRNA (orange peak). **D)** 3WJ-EpCAM-Alexa 647 nanoparticle internalization by confocal microscopy. Nucleus was stained with DAPI, F-actin was stained with Phalloidin (Alexa 488) dye, and nanoparticle was conjugated with Alexa 647. **E)** Qualitative and quantitative analysis of the D5D protein level through the Western blot analysis after 3WJ-EpCAM-D5D siRNA nanoparticle treatment. In control, relative expression of D5D to β -Actin. **F)** Immunocytochemistry analysis to determine efficacy to knockdown D5D by 3WJ-EpCAM-D5D siRNA. 3WJ-EpCAM (nanoparticle without therapeutic moiety) was serving as a control, and 3WJ-EpCAM-D5D siRNA nanoparticle was acting as a treatment. Data are expressed in terms of Mean $\pm$ SEM. n=4 for C-F. *** $p<0.001$, * $p<0.05$ vs. respective control.

Figure 2: *In vivo* effect of 3WJ-EpCAM-D5D siRNA / 3WJ-EpCAM-Alexa 647 nanoparticles in 4T1 induced orthotopic breast cancer model

A) Pictorial representation of the study plan to determine biodistribution of 3WJ-EpCAM-Alexa 647 nanoparticles. **B)** Pictorial representation of study plan to determine *in vivo* therapeutic effect of 3WJ-EpCAM-D5D siRNA nanoparticles and DGLA through 4T1 induced orthotopic breast cancer model. **C)** Bio-distribution analysis of 3WJ-Alexa 647 and 3WJ-EpCAM-Alexa 647 nanoparticles after 8hours of administration in various organs. Animals in control group were administered saline, animals in EpCAM negative group were provided 3WJ-Alexa 647, and animals in treatment group were administered 3WJ-EpCAM-

Alexa 647 nanoparticle. **D)** Fold change in tumor size analysis with respect to time. During the treatment period tumor size was measured by digital Vernier caliper. At the beginning of treatment (Day 10) tumor size in each group was considered as one-fold. **E)** Images of the harvested tumor after the treatment period. **F)** Immunohistochemistry analysis of D5D protein in tumor after the treatment period. DAPI was used to stain the nucleus and Alexa 633 were used to indicate D5D. n=3 for C and F and n=4 for D and E. ** $p<0.01$ vs. Vehicle.

Figure 3: *In vivo* cancer growth inhibitory effect of combination of 3WJ-EpCAM-D5D siRNA nanoparticle and DGLA in 4T1 induced orthotopic breast cancer model.

A) GC/MS analysis of production of an anti-cancer metabolite, 8-HOA, by different treatment from tumor tissue. **B)** LC-MS analysis of AA generated from 4T1 induced tumor after different treatment. **C)** LC-MS analysis of DGLA/AA ratio from tumor tissue. **D)** HDAC activity analysis from tumor tissue after different treatment. **E)** Qualitative and quantitative Immunohistochemistry analysis of tumor tissue for C. PARP and Ki-67. DAPI was used to stain nucleus and respective primary and Alexa 633 (Secondary) were used to stain C. PARP and Ki-67. **F)** Qualitative and quantitative Western analysis of apoptotic proteins after different treatment. n=3 for A-C and E-F and n=4 for D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Vehicle.

Figure 4: *In vivo* anti-metastatic effect of combination of 3WJ-EpCAM-D5D siRNA nanoparticle and DGLA in 4T1 induced orthotopic breast cancer model

A) Pictures of lung highlighted (arrow) with a representative metastatic lung nodule after different treatment. **B)** H&E staining of paraffin-embedded lungs represent metastatic nodule (dotted circle) in Vehicle, DGLA, 3WJ-EpCAM-D5D siRNA, and combination of DGLA and 3WJ-EpCAM-D5D siRNA treated group. **C)** Qualitative and quantitative analysis of metastatic proteins by the Western blot analysis. **D)** Immunohistochemistry analysis of tumor tissue stained with E-cadherin, MMP-2 and Vimentin. DAPI was used to stain nucleus and respective Primary and Alexa 633 (Secondary) were used to stain E-

cadherin, MMP-2 and Vimentin. n=4 for A and B, n=3 for C and D. * $p<0.05$ and ** $p<0.01$ vs. Vehicle.

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