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Lipidome-based Targeting of STAT3-driven Breast Cancer Cells Using Poly-L-glutamic acid-coated Layer-by-Layer Nanoparticles

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Abstract

The oncogenic transcription factor STAT3 is aberrantly activated in 70% of breast cancers, including nearly all triple-negative breast cancers (TNBC). Since STAT3 is difficult to target directly, we considered whether metabolic changes driven by activated STAT3 could provide a therapeutic opportunity. We found that STAT3 prominently modulated several lipid classes, with most profound effects on N-acyl taurine and arachidonic acid, both of which are involved in plasma membrane remodeling. To exploit these metabolic changes therapeutically, we screened a library of layer-by-layer (LbL) nanoparticles (NP) differing in the surface layer that modulates interactivity with the cell membrane. We found that poly-L-glutamic acid (PLE)-coated NPs bind to STAT3-transformed breast cancer cells with 50% greater efficiency than to non-transformed

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cells, and the heightened PLE-NP binding to TNBC cells was attenuated by STAT3 inhibition. This effect was also observed in densely packed three-dimensional breast cancer organoids. As STAT3-transformed cells show greater resistance to cytotoxic agents, we evaluated if enhanced targeted delivery via PLE-NPs would provide a therapeutic advantage. We found that cisplatin-loaded PLE-NPs induced apoptosis of STAT3-driven cells at lower doses compared to both unencapsulated cisplatin and cisplatin-loaded non-targeted NPs. In addition, since radiation is commonly used in breast cancer treatment, and may alter cellular lipid distribution, we analyzed its effect on PLE-NP-cell binding. Irradiation of cells enhanced the STAT3-targeting properties of PLE-NPs in a dose-dependent manner, suggesting potential synergies between these therapeutic modalities. These findings suggest that cellular lipid changes driven by activated STAT3 may be exploited therapeutically using unique LbL nanoparticles.

Keywords

breast cancer; STAT3; lipidomics; targeted drug delivery; Layer-by-Layer nanoparticles

Introduction

Breast cancer is the most prevalent malignancy and the second leading cause of cancer-related deaths in women, with an estimated 271,000 newly diagnosed patients in 2019 in the United States (1). Triple-negative breast cancer (TNBC) is the most aggressive type of breast cancer, characterized by poor overall survival, substantial metastatic potential, and high recurrence rate (2). Unlike the other subtypes, TNBCs currently lack targeted forms of therapy and are commonly treated with non-specific methods such as chemotherapy, radiation and surgery. Along with limited efficacy, non-targeted approaches are associated with significant toxicity, emphasizing the need for novel treatment strategies (3).

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that plays a pivotal role in regulating the expression of genes involved in cell survival, proliferation, migration, malignant transformation and immune response (4). Under basal conditions, STAT3 resides in the cytoplasm in an inactive conformation. In response to cytokines and growth factors, STAT3 becomes phosphorylated on a single tyrosine residue (tyr705) by tyrosine kinases, such as Janus kinases (JAK). Upon activation by phosphorylation, STAT3 forms an active dimer via Src Homology 2 (SH2) domain and translocates to the nucleus where it binds a nine base pair sequence in the promoter of target genes. In normal cells, STAT3 function is tightly regulated by endogenous inhibitors allowing its rapid and transient physiological activation in response to external stimuli. However, breast cancer cells, including TNBC, are commonly characterized by constitutive activation of STAT3, resulting in the overexpression of genes that promote malignant behavior. In addition, breast cancer cells are often dependent on continual STAT3 signaling for survival (5); thus, targeting STAT3 represents a promising therapeutic strategy for the treatment of such cancers. However, since transcription factors like STAT3 have large and relatively flat molecular surfaces to allow protein-DNA and protein-protein interactions, it makes it challenging to directly target these proteins using small molecules (6). Therefore, we investigated whether

metabolic changes driven by constitutive STAT3 activation could provide a therapeutic opportunity in targeting STAT3-transformed malignant cells.

Materials and Methods

Cell lines.

MDA-MB-231 and MDA-MB-468 cells (kindly provided by Myles Brown, Dana-Farber Cancer Institute) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. SUM159PT cells (received from Kornelia Polyak, Dana-Farber Cancer Institute) were maintained in Ham's F-12 medium supplemented with 5% heat inactivated fetal bovine serum, 10 mM HEPES (Gibco), 1 µg/mL hydrocortisone (Sigma) and 5 µg/mL insulin (Sigma). MCF-10A cells (ATCC, cri-10317) were stably engineered to express STAT3C (with a FLAG epitope) under an inducible promoter, and were maintained in DMEM/F12 containing 5% horse serum, 20 ng/mL EGF (Peprotech, Rocky Hill, NJ), 0.5 µg/mL hydrocortisone (Sigma), 100 ng/mL cholera toxin (Sigma) and 10 µg/mL insulin (Sigma). To induce STAT3C expression, cells were incubated with 2 µg/mL doxycycline (Sigma) for 24 hours prior to treatment with nanoparticles. Cells were maintained in a humidified incubator at 37°C with 5% CO₂ and passaged for less than three months after thawing. All cells were authenticated by short tandem repeat DNA profiling, and were routinely tested for the presence of *Mycoplasma* by PCR.

Immunoblot analyses.

Cells for immunoblot analyses were lysed on ice for 15 minutes in RIPA lysis buffer (Boston BioProducts) with phosphatase inhibitor cocktail (Cell Signaling Technology) and complete protease inhibitors (Roche). Immunoblots were probed with antibodies to phospho-STAT3 (Tyr705) (9131, Cell Signaling Technology), STAT3 (sc-482, Santa Cruz), FLAG (F1804, Sigma), tubulin (T5168, Sigma-Aldrich).

mRNA Expression Analyses (RT-PCR).

Total cellular RNA was isolated using Qiagen RNeasy Mini kits. RNA quality was evaluated on a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific), and reverse transcribed with TaqMan (Applied Biosystems) to generate cDNA. Quantitative polymerase chain reaction (qPCR) was performed in quadruplicate using Power SYBR master mix (Applied Biosystems) on a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). Specificity of amplification was confirmed by melt curve analysis. Cycle threshold (C_t) values for target genes were normalized to the endogenous reference gene GAPDH, and the fold change was determined by dividing the expression in each sample by that of the untreated control sample. Primer sequences (Supplementary Table 1) were designed from the UCSC genome browser reference mRNA sequences using Primer3.

Nanoparticles.

The LbL nanoparticle library was synthesized and characterized as previously described (7,8). Passive loading was employed for cisplatin (CDDP) encapsulation as described in the Supplementary Materials and Methods (9,10).

RNA interference.

Cells were transfected using Lipofectamine RNAiMAX with 10 nM of siRNA targeting STAT3 #2 or STAT3 #3 (D-003544-02-0010 and D-003544-03-0010, respectively; Dharmacon), or non-targeting siRNA control (sc-37007, Santa Cruz). Cells were transfected with siRNA for 48 hours, after which the media was changed to remove lipofectamine, and the cells were then incubated for an additional 24 hours (MDA-MB-231) or 72 hours (SUM159PT) prior to nanoparticle treatment.

Drug treatment.

MCF-10A cells were pre-incubated with 5 μ M pyrimethamine (46706, Sigma Aldrich) or 2.5 μ M ruxolitinib (ab141356, Abcam) for 4.5 hours. They were then induced with doxycycline (or vehicle control) for 24 hours, prior to immunoblot and qRT-PCR analyses or nanoparticle treatment. When evaluating the mode of cellular death in response to drug-loaded NP treatment, the cells were treated as previously described to induce or inhibit STAT3, then incubated for 1 hour with 3 μ M ferrostatin-1 (SML0583, Sigma Aldrich) or 20 μ M necrostatin-1 (N9037, Sigma Aldrich). The cells were then treated with the indicated concentration of CDDP-loaded NPs and analyzed as described below.

Mass spectrometry-based lipidomic profiling.

Prior to mass spectrometry analysis, MDA-MB-468 cells were transfected with siRNA targeting STAT3 #3 or non-targeting siRNA control as previously described, or treated with 10 μ M pyrimethamine (or DMSO control) for 24 hours, and MCF-10A cells were treated for 24 hours with 2 μ g/mL DOX (or DMSO control). Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was performed with five replicates per condition using an electrospray ionization (ESI) source on an Agilent 6430 or 6460 QQQ LC-MS/MS (Agilent Technologies), as described in detail in Supplementary Materials and Methods (11,12).

Nanoparticle treatment.

After the indicated treatments, cells were incubated with 100 ng/mL fluorescently-labeled nanoparticles for 24 hours prior to flow cytometric analysis or microscopic imaging. Following treatment with CDDP-loaded nanoparticles or free CDDP, the cells were washed with PBS, replenished with fresh media and incubated for a total of three days, after which viability was detected by flow cytometry.

Nanoparticle library screening.

MCF-10A cells were incubated with doxycycline (to induce STAT3C) or vehicle control for 24 hours, after which the membrane was stained red using Wheat Germ Agglutinin (W11262, Life Technologies) and nuclei were stained blue using Hoechst stain (4082S, Cell Signaling Technology). The stains were washed with phosphate-buffered saline (PBS) and the cells were treated with 100 ng/mL of different coating nanoparticles and imaged on CellObserver (Zeiss) every 30 minutes for a total of 8 hours. The membrane and nuclear binding were analyzed with custom pipelines (Supplemental Materials and Methods) created in CellProfiler 2.2.0 software (Broad Institute of Harvard and MIT).

Flow cytometry.

Cells were induced with doxycycline (MCF-10A) or transfected with siRNA (SUM159PT and MDA-MB-231), and treated with fluorescently-labeled non-loaded or CDDP-loaded nanoparticles as described above. After incubation with nanoparticles, the cells were washed with PBS, trypsinized and neutralized with corresponding media, and washed again with PBS.

Analyzing the cellular binding of non-loaded nanoparticles.—Cells were resuspended and incubated with live/dead stain (L34975, Life Technologies) for 30 minutes at 4°C in the dark. The stain was removed, and the cells were resuspended in PBS containing 3% FBS, when analyzed. The NP-cell binding was measured by yellow-green fluorescence of the nanoparticles in the population of viable single cells.

Analyzing viability after CDDP-loaded NP treatment.: Apoptosis was detected by Annexin V/4',6-diamidino-2-phenylindole (DAPI) staining, which was performed using the Apoptosis Detection Kit I (BD Biosciences).

All of the samples were prepared in triplicate, and each experiment was performed with at least two independent biological replicates. Fluorescence distribution was acquired on a BD LRSFortessa instrument and analyzed with FlowJo software (Treestar).

Three dimensional organoid culture.

Cells were cultured for three to ten days in Nanoculture plates (MBL International) using the recommended culturing protocol. To preserve STAT3C induction, the MCF-10A cells were replenished with 2 µg/mL doxycycline (or DMSO control) every three days of culturing. Organoids were stained with 2 µM Lox1 hypoxia stain (#Lox1, MBL International) and treated with 100 ng/mL nanoparticles for 24 hours prior to confocal microscopy imaging using Zeiss LSM 880+ Airyscan microscope. Yellow-green (NP) and red (Lox1) fluorescence were quantitated with a custom macro (Supplemental Materials and Methods) written in ImageJ software (National Institutes of Health). Organoid images, Z-stack videos, and XYZ projection were prepared using ImageJ software, and video presentations of the NP distribution across three dimensions were prepared using Vision4D 3.1 software (arivis AG, Germany).

Deconvolution microscopy.

The preparation of samples is described in detail in the Supplementary Materials and Methods. Briefly, MCF-10A cells were seeded in microscope slide chamber with DOX- or DMSO-containing media for 24 hours, followed by PLE-NP treatment for an additional 2 or 24 hours (n=3). They were fixed and stained for nucleus and membrane using Hoechst stain (blue) and Wheat Germ Agglutinin (red), respectively. The cells were imaged on an Inverted Olympus X71 microscope and processed using OMX softWoRx software (Applied Precision/GE).

Gamma irradiation.

Cells were irradiated with a ^{137}Cs source as a single administration of the indicated dose. Relative viable cell number was determined after 1 to 3 days of incubation by measuring ATP-dependent bioluminescence (Cell Titer-Glo; Promega) on a Luminoskan Ascent luminometer. For NP-cell binding experiments, the cells were treated for 24 hours with 100 ng/mL PLE-NP after irradiation, after which NP-cell association was quantified with flow cytometry, as described above.

Gene set enrichment analysis.

GSE5460 breast cancer and GSE3526 normal tissue microarray datasets (13) were downloaded from the Gene Expression Omnibus. Gene-set enrichment analysis was performed using xapps.gsea from the Broad Institute of MIT and Harvard (<http://software.broadinstitute.org>), based on STAT3 gene expression signatures (14).

Statistical Analyses.

Results are presented as mean $\pm$ SE unless otherwise indicated. Two-tailed Student t tests for paired samples were performed with GraphPad Prism 8.4 software (La Jolla, CA). Values of $p < 0.05$ were considered significant (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

Results

STAT3 transcriptional activity modulates cellular lipid profile in breast cancer cells.

STAT3 is aberrantly activated in the majority of breast cancers and represents a crucial mediator of the pathogenesis of this disease. Whereas STAT3 protein is ubiquitously expressed in normal tissue, its transcriptional activity differs greatly between normal and breast cancer tissue (Figure 1A), indicating that STAT3-directed treatment approaches would be associated with a favorable therapeutic index. Since STAT3 is difficult to target directly, we investigated whether metabolic changes driven by constitutive activation of STAT3 could provide a vulnerability in breast cancer cells that can be exploited therapeutically. Recognizing that malignant transformation is associated with changes in a variety of cellular metabolites, we examined how STAT3 qualitatively and quantitatively affects lipid distribution in mammary epithelial cells using two complementary systems. First, we used non-transformed MCF-10A cells in which an activated form of STAT3 can be expressed with an inducible doxycycline (DOX)-responsive promoter (Figure 1A), and is sufficient to cause tumorigenic properties (15). Induction of the promoter increased STAT3 protein expression and its required tyrosine phosphorylation, followed by enhanced mRNA expression of target genes including SOCS3, BCL3, BCLAF1, and a positive autoregulation of its own expression (Figure 1B). We also used a triple-negative breast cancer cell line, MDA-MB-468, characterized by constitutive STAT3 tyrosine phosphorylation, in which we inhibited STAT3 activity genetically (with RNA interference) or pharmacologically (with the small molecule inhibitor pyrimethamine (PYR) (Figure 1C) (16)). We then performed liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)-based profiling to evaluate a total of 220 lipid molecules (Supplementary table 2). We found several classes of

lipid molecules that were significantly changed by activated STAT3 (Figures 1D and E). Four distinct but similar N-acyl-aurine (NAT) metabolites were decreased in MDA-MB-468 cells with activated STAT3. Similarly, the presence of N-arachidonoyl taurine (C20:4 NAT), which has been reported to induce apoptosis of prostate cancer cells (17,18), was diminished when STAT3C was induced in MCF-10A cells. Two other taurine derivatives, N-palmitoyl (C16:0) and N-stearoyl (C18:0) taurine, were strongly reduced when STAT3 is constitutively active in MDA-MB-468 cells, and showed an analogous trend with STAT3C induction. Interestingly, several lipid metabolites that were negatively modulated by STAT3, including taurine, were conjugated with arachidonic acid (AA, C20:4), the precursor for eicosanoid synthesis (Figures 1D and E). Both taurine and AA metabolites are actively involved in plasma membrane remodeling (19,20), providing a potential molecular distinction of STAT3-driven cancer cells.

LbL nanoparticle library screen to identify STAT3-targeting drug carriers.

Given these prominent changes in lipid composition driven by activated STAT3, we investigated targeting STAT3-driven cancer cells using novel nanoparticles that can exploit these differences. Layer-by-layer (LbL) nanoparticles (NP) consist of a carboxy-modified latex colloidal core (CML), surrounded by multiple ultrathin alternatively charged layers. These layers are composed of cationic poly-L-arginine (PLR) with polyanions as the terminal layer, containing either sulfated or carboxylated functional groups. The average hydrodynamic diameter of all NPs range from 100 to 155 nm (7,8). Different charge layers allow packaging of a variety of therapeutic molecules with controlled release of the active substance (21,22). Most importantly, the surface layer is responsible for the NP interaction with the plasma membrane, which enables the design of LbL NPs with tumor-targeting properties (7,23). Therefore, we screened a library of 12 LbL NPs differing in their external layer to determine the surface composition that allowed the highest binding to cells characterized by the STAT3-induced lipid profile. MCF-10A cells with and without STAT3C induction were treated with distinct NPs, after which their membrane and nuclear co-localization were recorded every 30 minutes for a total of 8 hours (Figure 2A, Supplementary Figure 1 and Supplementary Figure 2). We found that the poly-L-glutamic acid (PLE) coated NPs showed significantly greater binding to STAT3-driven cells compared to non-transformed counterparts (Figures 2A–C). This did not reflect a non-specific effect of neoplastic transformation, as LbL NPs coated with other modifications showed no difference in cell binding affinity (Figure 2A and Supplementary Figure 1). The differential PLE-NP-cell interaction was detected within the plasma membrane, and co-localization with nuclei was minimal for all investigated NPs in both conditions (Supplementary Figure 2). To further characterize the PLE-NP interactions with MCF10A cells with and without STAT3C induction we performed deconvolution microscopy (Figure 2D and Supplementary Figure 3). Whereas PLE-NPs associated to a low extent with non-transformed MCF-10A cells, induction of STAT3 led to recruitment of considerably greater numbers of these NPs (Figure 2D and Supplementary Figure 3), with targeting affinity being larger than projected from the initial screening data. This variability may have arisen from differences in cell culture density, as well as alterations in quantitation from single cell analysis compared with mean cell binding over a population (as in Figures 2A, 2B, and 2C). To address these quantitative differences in cellular binding, we further conducted flow cytometric analyses. As PLE-NPs

have previously been shown to accumulate in tumor tissue *in vivo*, while having attenuated distribution in vital organs (7), we evaluated targeting breast cancer cells based on their abnormal STAT3 activity using PLE-coated nanoparticles.

PLE-NPs preferentially target STAT3-driven breast cancer cells.

To quantify the differential cell binding of PLE-NPs in breast cancer cells, we first used MCF-10A cells with doxycycline-inducible expression of STAT3C (Figure 3A). Using quantitative flow cytometry, we found that these nanoparticles preferentially associate with the cells with activated STAT3, displaying 50% greater cell-associated nanoparticle fluorescence compared to their non-transformed counterparts (Figure 3B). This effect was specific to PLE-NPs, as NPs with other modifications, such as dextran sulfate (DXS), did not reveal a similar effect (Figure 3B). To independently assess whether these differences were STAT3-dependent, we inhibited STAT3 phosphorylation with the Jak kinase inhibitor ruxolitinib (RUX) or STAT3 transcriptional activity with pyrimethamine (Figure 3C). Both treatments led to a 50% reduction in cell-associated nanoparticle fluorescence.

Since STAT3 is activated under basal conditions in most TNBCs, we investigated whether PLE-NPs could be used as a STAT3-targeted approach for this subset of breast cancers. We used the TNBC cell lines SUM159PT and MDA-MB-231 cells, as they both show constitutive tyrosine phosphorylation of STAT3 (Figure 3D). Furthermore, these cells are dependent on STAT3 for long term survival, as it was not possible to derive STAT3 null clones of these lines using CRISPR/Cas9. Therefore, we performed RNA interference-mediated silencing in these systems to determine STAT3-dependent interactions with NPs (Figures 3E and F). We found that the TNBC cell lines MDA-MB-231 and SUM159PT showed rapid binding of PLE-NPs, which was attenuated by depletion of STAT3 using two different siRNAs. This mechanism was specific for PLE-NPs, as DXS-coated NPs displayed either no difference (or showed increased accumulation) on cells lacking activated STAT3 (Figures 3G and H).

PLE-NP penetrate three-dimensional mammary cell organoids.

To better model behavior in tumor systems, we characterized PLE-NP accumulation in three-dimensional MCF-10A cell organoids, which more closely recapitulate the architecture of a tumor *in vivo* (24,25). STAT3 activation promoted cellular growth and proliferation, resulting in the formation of a markedly greater number of large colonies (Figure 4A). STAT3-transformed organoids also grew at much higher cellular density, which further resulted in greater hypoxia within the organoids (Figures 4B–D). To determine the penetration and distribution of NP within the organoid structure, we imaged the NP fluorescence across three dimensions (Figure 4C). In addition to occupying the surface of the cellular structures, PLE-NPs were clearly able to penetrate deep inside of the organoids, as seen in Z-stack and 3D organoid videos (Supplemental Figures 4 and 5) and XYZ organoid projection (Figure 4E). Furthermore, we quantified the NP fluorescence across three dimensions and found that the mean organoid fluorescence was substantially greater in organoids with activated STAT3 than in those without (Figures 4C and F). Recognizing that more voluminous organoids have greater surface area in direct contact with the NP-containing media, we also assessed the mean fluorescence per unit volume of the organoid.

Despite the substantially higher cellular density of STAT3-transformed organoids, which might have been expected to impede the penetration of NPs, these organoids showed greater quantitative NP fluorescence per organoid (by 40%) as well as per organoid volume (by 73%) (Figure 4F). Thus, even in tightly packed three-dimensional organoids, PLE-NPs show preferential penetration and intra-organoid accumulation in the presence of activated STAT3.

STAT3-driven breast cancer cells show preferential sensitivity to cisplatin delivered via PLE-NPs.

To further evaluate the translational utility of these findings, we tested the effect of PLE-NPs incorporating the cytotoxic agent cisplatin (CDDP), as platinum agents are clinically used in TNBC treatment (26,27). Induction of STAT3 rendered MCF-10A cells more vulnerable to CDDP-loaded PLE-NPs and significantly increased cell death, whereas MCF-10A cells without activated STAT3 showed greater viability after such treatment (Figure 5A and Supplemental Figure 6). Similarly, the TNBC cell line SUM159PT showed increased apoptotic sensitivity to CDDP delivered via PLE-NPs, and this effect was attenuated when STAT3 was depleted with siRNA (Figure 5B and Supplemental Figures 7A and B). This did not reflect a general increased sensitivity to cell killing in the cells with activated STAT3, as these cells were overall less sensitive or generally resistant to free CDDP treatment (Supplementary Figure 6 and Figures 5A and B). Similarly, CDDP-loaded NPs that lacked LbL coatings or were coated with non-targeting DXS showed less cytotoxic effect on the cells with activated STAT3 (Figure 5A). Triple-negative SUM159PT cells displayed particular resistance to non-targeted forms of CDDP, as each of the tested conditions induced little apoptotic effect (Figure 5B), even with both prolonged incubation time and high treatment concentrations (Supplemental Figure 7A–D). Overall, these data suggest that drug-loaded PLE-NPs could be an effective targeted therapy for STAT3-driven breast cancers, including TNBC, while minimizing toxic effects to normal cells characterized by low and transient STAT3 activation.

Since cell death induced by CDDP-loaded NPs leads to early positivity of annexin V staining (without loss of membrane integrity), cell death induced by these agents is clearly, at least in part, mediated by apoptosis. However, given the rapid cell death induced by these NPs, as well as the possible clinical implications of the particular mode of cell death induced, we examined this aspect in more detail. STAT3 function has been linked with both ferroptosis and necroptosis (28,29). Therefore, we tested the effects of ferroptosis and necroptosis inhibitors (ferrostatin-1 and necrostatin-1, respectively) on cell death induced by CDDP-loaded PLE-NP. We did not observe any significant difference in cell killing in any system when incubating the cells with these inhibitors prior to CDDP-loaded NP treatment (Supplemental Figure 8). While it is possible that other modes of cell death may contribute to killing mediated by CDDP-loaded PLE-NPs, it appears that apoptosis is a prominent component of the mechanism.

Gamma radiation increases targeting properties of PLE-NPs.

Gamma radiation is commonly used in the treatment of breast cancer, including TNBC, and is known to alter the properties of the plasma membrane (30). Therefore, we tested whether radiation could impact PLE-NP-cell interaction in a STAT3-dependent manner. To exclude

effects of widespread cell death, we first assessed viable cell number of STAT3-transformed and untransformed MCF-10A cells treated over a range of radiation doses (Supplementary Figure 9). We found that doses below 6 Gy were effective at avoiding a complete proliferation arrest. Next, we evaluated the effect and dose dependency of acute gamma irradiation on PLE-NP cell binding (Figure 5C). Interestingly, gamma radiation reduced the association of PLE-NPs with non-transformed MCF-10A cells in a dose dependent manner. However, binding to STAT3-transformed MCF-10A cells was minimally affected. Consequently, the difference in cell-associated PLE-NP fluorescence between non-transformed and STAT3-transformed cells increased from approximately 40% to 80% following 5 Gy irradiation (Figure 5C). These findings suggest a potential synergistic effect between radiation and nanoparticle-based targeting of breast cancer cells.

Discussion

Previous studies have suggested a crosstalk between STAT3 signaling and lipid metabolism, including lipolysis, beta oxidation, ferroptosis and membrane lipid raft modeling in various cancer types (29, 31–33). While such lipid changes may underlie alterations in morphology, motility, and invasion of STAT3-transformed cells, comprehensive evaluation of lipid metabolites modulated by activated STAT3 was lacking. Using an unbiased strategy employing complementary breast cancer models, we identified several metabolites modulated by activated STAT3, including a profound reduction of cellular N-acyl taurine derivatives and AA content. We also detected STAT3 regulation of other metabolites, such as triacylglycerols and phosphatidylinositol derivatives; however, this modulation was not observed consistently across all of the tested models. While these changes may be important, they may also be more likely to be cell context dependent.

These consistent and large decreases in AA and taurine may be directly related to transcriptional changes mediated by activated STAT3. Gene set enrichment analysis (GSEA) using microarray data from 129 primary breast cancer patient samples (13) showed that STAT3 gene expression signatures are highly enriched in patient samples with low expression of both cystathionine gamma lyase (CTH) and cysteine dioxygenase (CDO1), the two enzymes required for taurine synthesis from homocysteine (Figures 6A and B and Supplementary Figures 10A and B). Taurine plays a role in various cellular processes exerting antioxidant, membrane stabilizing, and anti-inflammatory effect (34), with tumor suppressing activity reported in several types of malignancies (35). Whereas taurine inhibition of STAT3 phosphorylation has been described (36,37), our findings suggest that activated STAT3 may reduce taurine biosynthesis through transcriptional regulation of CTH and CDO1 enzymes.

With regard to AA cellular content, we evaluated the correlation between STAT3 gene expression signatures and the expression of enzymes involved in AA metabolism. STAT3 gene expression signatures were highly associated with mRNA expression of AA catabolic enzymes cyclooxygenase 2 (COX2, PTGS2) and 5-lipoxygenase (5-LOX, ALOX5) (Figures 6C and D and Supplementary Figures 10C and D). By contrast, phospholipase A2 (PLA2G4A), which is necessary for AA membrane release, did not show a significant relationship (Supplementary Figure 10E). Similarly, these STAT3 signatures showed no

correlation with the housekeeping gene GAPDH in the same breast cancer patient datasets (Supplementary Figure 10F and G). Consistent with previous findings (38–40), these data indicate an intriguing crosstalk between the STAT3 signaling pathway and enhanced eicosanoid synthesis associated with inflammatory processes and cancer progression (30). The fact that both taurine derivatives and AA are involved in plasma membrane remodeling (19,20) provides a basis for how changes in the metabolic architecture resulting from aberrant STAT3 activation can provide specificity for a tumor-targeting approach employing surface-directed nanoparticles.

Considering the importance of STAT3 signaling in the pathogenesis of breast cancer and other common tumors, and the high potential therapeutic index of blocking STAT3 transcriptional function, a number of strategies have been taken to identify inhibitors of this protein (41–45). Several unbiased screening strategies have been used to identify STAT3 inhibitors. For example, the use of a computational screen for potential STAT3 transcriptional inhibitors based on a STAT3 gene expression signature led to the identification of atovaquone, an FDA approved anti-microbial drug, which decreased gp130-dependent phosphorylation of STAT3 (43). Chemical library screens for specific inhibitors of STAT3-dependent gene expression revealed nifuroxazide and pyrimethamine as potent STAT3 transcriptional inhibitors (16,44). These findings have led to ongoing clinical trials of pyrimethamine and atovaquone for the treatment of hematological malignancies and lung cancer. Targeted strategies for the inhibition of STAT3 have included the development of antisense oligonucleotides and molecules designed to bind to specific functional regions of STAT3, including the SH2 and DNA-binding domains (42,45). It remains to be seen whether these strategies will translate into clinically useful agents for on-target inhibition of STAT3 (45). While many of these approaches hold promise, this is the first study to our knowledge that targets cancer cells based on exploiting STAT3-driven changes in cellular lipid composition.

Our LbL nanoparticle library screen revealed that anionic liposomes coated with PLE as the terminal layer preferentially bind to cells with constitutive STAT3 activation, including TNBC cell lines (Figures 2 and 3). This property was unique for PLE-NPs, as NPs with other surface coatings did not show a similar effect (Figure 2). Furthermore, abrogation of STAT3 mRNA expression, phosphorylation, or transcriptional activity attenuated cell binding of PLE-NPs, suggesting the specificity of targeting STAT3-driven cells by these nanosystems (Figure 3). Tumor-targeting properties of PLE-NPs had previously been described in ovarian cancer models, although, the underlying biological mechanism for this effect was unclear (7). As STAT3 is commonly aberrantly activated in ovarian cancer, we evaluated the extent to which this targeting ability was driven by activated STAT3. We compared the previously reported PLE-NP binding affinity in these ovarian cancer cell lines with mRNA expression of the two target genes most specific for STAT3 transcriptional activity, SOCS3 and JUNB (Supplemental Figure 11). Overall, all of the tested ovarian cell lines showed greater PLE-NP recruitment than other investigated NPs and than non-malignant cells (7) (Supplemental Figure 11B and C). These cell lines also expressed relatively high levels of the STAT3-regulated genes compared with the other ovarian cell lines. Additionally, cell lines such as JHSO2, COV318, FUOV1, SKOV3 and COV362 showed a positive correlation between PLE-NP binding affinity and STAT3 functional

activation. These analyses provide independent confirmation, in a different tumor system, of the affinity of PLE-NPs to tumor cells with activated STAT3.

In addition, high resolution microscopy suggests that these nanoparticles bind and potentially integrate within the plasma membrane (7) (Supplemental Figure 3), further supporting the importance of cellular lipid composition in their targeting specificity. Although they may not necessarily be internalized into the cytoplasm, PLE-coated NPs retain the ability to accumulate preferentially in tumor tissue *in vitro* and *in vivo* (7,46,47). Furthermore, their ability to penetrate deep into the dense cellular structures of STAT3-transformed organoids while retaining targeting affinity (Figure 4 and Supplemental Figures 4 and 5), represents an important feature supporting further *in vivo* investigation. Consistent with previous reports (46,47), we found they can preferentially deliver effective therapeutic payloads to malignant cells in a more efficient and specific manner compared to free drug (Figure 5).

The therapeutic relevance of this finding is reflected in the strong cytotoxic effect of CDDP delivered by PLE-NPs to STAT3-driven cells (Figure 5). On the contrary, cells lacking constitutive STAT3 activity show greater survival after this treatment, suggesting a strong therapeutic index. Furthermore, constitutive STAT3 signaling is implicated in aggressive malignant behavior and the development of chemo-resistance patterns in various malignancies, likely through increased expression of pro-survival genes (48). Accordingly, STAT3-transformed cancer cells show greater resistance to free CDDP and drug-carrying non-targeted NPs, whereas TNBC cells display substantial resistance to non-targeted forms of treatment (Figure 5B and Supplemental Figure 7). These findings further emphasize the relevance of the specific cytotoxic effect of PLE-NP-mediated delivery of CDDP, which presumably delivers a higher concentration of drug that can overcome these effects. To try to quantitate this effect further, we analyzed intracellular platinum concentration in the MCF-10A cell model treated with PLE-NP-encapsulated or free CDDP, using inductively coupled plasma mass spectrometry (ICP-MS; Galbraith Laboratories Inc., Knoxville, TN). Unfortunately, the platinum concentration in all of the tested samples fell below the quantification limit, precluding a definitive answer to this question.

LbL NPs are designed to be biocompatible and have high stability when administered *in vivo*, including a favorable pharmacokinetic profile (49). The affinity of PLE-NPs for STAT3-driven tumor cells could further minimize their off-target effects, facilitating translational and clinical development. Taken together with the low STAT3 activation profile in normal cells (Figure 1A), a STAT3-targeted approach that utilizes PLE-NPs might provide a more efficient and safe module for drug delivery of cytotoxic agents. Finally, radiation treatment is an important component of breast cancer management (2,3). Irradiation is known to alter the properties of cellular and plasma membrane lipid composition, and has been linked to the induction of enzymes that produce inflammatory lipids, including COX2 and 5-LOX (28). We found that irradiation led to suppressed binding of PLE-NP to non-transformed cells, while minimally affecting binding to STAT3-transformed cells (Figure 5C). Although the exact mechanism of this phenomenon remains to be elucidated, one potential hypothesis is that lipid changes driven by activated STAT3 may be more stable than those in non-transformed cells. Given the role of STAT3 in development of resistance to

gamma radiation (50), STAT3 activity might curtail the radiation-induced lipid changes and consequently maintain high binding levels of PLE-NPs to STAT3-transformed cells. On the other hand, cells lacking activated STAT3 might undergo greater lipid changes following radiation, resulting in reduced binding of PLE-coated nanosystems. These findings raise the possibility of an enhanced therapeutic index in combining PLE-NP-based drug delivery with radiation therapy for TNBC.

In conclusion, LbL nanoparticles with a terminal poly-L-glutamic acid layer represent a new class of drug carriers that preferentially interact with STAT3-transformed mammary epithelial cells, and may offer synergy with radiation therapy. These findings provide a promising starting point for the development of a rational, targeted approach to treating triple-negative breast cancers, which currently lack such therapeutic options.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. STAT3 is activated in breast cancers and modulates lipid profiles in breast cancer cells. A. mRNA expression of STAT3 target genes is presented using microarray data of normal tissue (GSE3526, n=353) and breast cancer tissue (GSE5460, n=129). The heatmap was generated using GSEA software. B. MCF-10A cells were incubated with DOX (or DMSO control) for 24 hours to induce STAT3C and (C) MDA-MB-468 cells were incubated with 10 μ M PYR for 24 hours (or DMSO control) or transfected with siRNA to STAT3#3 (or non-targeting siRNA control) to inhibit STAT3 activity. Then the mRNA expression of endogenous STAT3 target genes was analyzed by qRT-PCR (left, n=4), and STAT3 protein expression and phosphorylation were detected by immunoblot analysis (right). D, E. STAT3 was inhibited in MDA-MB-468 cells using siSTAT3 (or non-targeting siRNA control) or PYR (or DMSO control), and STAT3C was induced in MCF-10A cells using DOX (or DMSO control) (n=5). Then a total of 220 lipid metabolites were analyzed by LC-MS/MS.

Changes in negatively (D) and positively (E) charged metabolites are shown as the fold change between active and inactive STAT3. *, p value < 0.05.

1. Nervous system (185); 2. Pituitary gland (8); 3. Spinal cord (8); 4. Bone marrow (5); 5. Lymph nodes (4); 6. Oral mucosa (4); 7. Tongue (8); 8. Salivary gland (4); 9. Esophagus (4); 10. Gastric (11); 11. Colon (3); 12. Liver (4); 13. Spleen (4); 14. Adipose tissue (10); 15. Thyroid gland (4); 16. Pharynx (4); 17. Tonsils (3); 18. Trachea (3); 19. Bronchus (3); 20. Lung (3); 21. Heart (7); 22. Coronary artery (3); 23. Saphenous vein (3); 24. Adrenal gland (4); 25. Kidney (8); 26. Urethra (3); 27. Cervix (4); 28. Endometrium (4); 29. Myometrium (5); 30. Ovary (4); 31. Vagina (4); 32. Vulva (4); 33. Breast (3); 34. Nipple (4); 35. Prostate (3); 36. Testes (3); 37. Skeletal muscle (5).

C16:0 – palmitic acid; C18:0 – stearic acid; C18:1 – oleic acid; C20:4 – arachidonic acid; NAT – N-acyl taurine; PIe – phosphatidylinositol; TAG – triacylglycerol; LPCe – lysophosphatidylcholine ether; SM – sphingomyelin; PSe – phosphatidylserine; MAGE – monoacylglycerol ether. “e” at the end of a fatty acid symbol indicates an ether linkage.

Figure 2. LbL nanoparticle library screen.

A–C. MCF-10A cells were incubated with DOX (or DMSO control) for 24 hours, after which they were stained and treated with a library of 12 NPs differing in the coating of the outer layer (n=2). NP-cell association was quantified using CellProfiler A. Heatmap of nanoparticle-cell association comparing the ratio in STAT3-transformed and non-transformed cells. B. Representative images of PLE-NP treated cells at indicated time points (n=2). C. Quantification of PLE-NPs co-localized with the cell membrane. D. Deconvolution microscopy of PLE-NP interaction with MCF-10A cells following 24 hour NP treatment, as shown by Z slice and Z projection. B, D. The membrane and nuclei were stained using Wheat Germ Agglutinin (red) and Hoechst stain (blue), respectively. Nanoparticle fluorescence is shown in green (B) or cyan (D).

DXS – dextran sulfate; HA – hyaluronic acid; PLD – poly-L-aspartic acid; Fuc – fucoidan; SBC – sulfated beta-cyclodextrin; HF – heparin sulfate folate conjugate; PAA – polyacrylic

acid; PLE – poly-L-glutamic acid; PLE-PEG - poly-L-glutamic block-polyethylene glycol; 1:1, 1:3 and 3:1 – indicated ratio blend of hyaluronic acid and poly-L-aspartic acid.

Figure 3. PLE-NPs preferentially target STAT3-driven breast cancer cells.

A. MCF-10A cells were treated with DOX (or DMSO control) to induce STAT3C, and protein expression was confirmed with the indicated immunoblots. B. Following treatment with the indicated NPs or controls, representative flow cytometry histograms are shown for the distribution of fluorescence (left) and quantification of median fluorescence intensity (right) (n=3). C. MCF-10A cells were pre-incubated for 4.5 hours with the STAT3 inhibitors pyrimethamine or ruxolitinib (or DMSO control), then STAT3C was induced with DOX (or DMSO control). The effects of the STAT3 pharmacological inhibitors were confirmed by qRT-PCR (left) and immunoblot analysis (center). Cells were then incubated with PLE-NP, and analyzed by flow cytometry (right). NP association with STAT3-transformed cells was normalized to the mean values of the non-transformed counterparts for each treatment (n=4). D. Immunoblot analysis of TNBC cell lines SUM159PT and MDA-MB-231 show constitutive phosphorylation of STAT3 on Y705. MDA-MB-231 (E) and SUM159PT (F)

cells were transfected with two different siSTAT3 (or non-targeting siRNA control), and the effect on STAT3 expression was determined by immunoblot. Cells were then treated with PLE-NP, non-targeting DXS-NP (or vehicle (H₂O) control), and analyzed by flow cytometry. Representative histograms of PLE-NP, DXS-NP and vehicle control fluorescence in MDA-MB-231 (G) and SUM159PT cells (H) are shown (left) and median fluorescent intensity was quantitated (right; n=3).

Figure 4. Characterization of PLE-NP accumulation in three-dimensional cell organoids. A,B. MCF-10A three-dimensional organoids were cultivated for 10 days with DMSO control (top) or DOX (bottom) to induce STAT3C expression. A. Organoid growth was imaged using optical microscopy and single organoid areas (top) and integrated densities (bottom) were quantified in ImageJ. B. Density of organoid cellular growth was recorded with confocal microscopy. C. Cells were cultured to form three-dimensional organoids in DMSO- or DOX-containing media for three days. Then they were treated with PLE-NPs and stained with Lox1 hypoxia stain for an additional 24 hours, then analyzed with confocal microscopy. Representative images of two independent experiments are shown (for DMSO, n=52; for DOX, n=70). D. Quantification of Lox1 hypoxia staining by ImageJ. E. XYZ projection of the PLE-NP distribution in DOX-induced MCF-10A organoid. F. The accumulation of the nanoparticles across the organoids was quantified using ImageJ and is

presented as mean fluorescence per organoid $\pm$ SEM (left) and mean fluorescence per organoid volume $\pm$ SEM (right).

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Figure 5. CDDP-loaded PLE-NPs show enhanced cytotoxic effects on STAT3-driven breast cancer cells.

Percent of viable MCF-10A (A) and SUM159PT cells (B) following treatment with CDDP-loaded PLE-NP, DXS-NP, non-coated liposomes, or free CDDP. A. Following STAT3C induction with DOX (or DMSO control), MCF-10A cells were treated for 2h with CDDP-loaded PLE-NPs, 4 hours with CDDP-loaded DXS- or non-coated NPs, or 24 hours with free CDDP. B. SUM159PT cells were transfected with two different siRNAs targeting STAT3 (or non-targeting siRNA control), then incubated for 24 hours with the indicated treatments. Cell survival was analyzed by flow cytometry using Annexin V/DAPI staining (n=3). C. MCF-10A cells were treated with DOX or DMSO, then irradiated with the indicated dose of gamma irradiation, and treated with PLE-NPs (or vehicle (H_2O) control) (n=4). NP-cell association was analyzed by flow cytometry, and the percent difference in NP-cell binding between non-transformed and STAT3-transformed cells is indicated for each of the radiation doses.

Figure 6. Expression of enzymes involved in taurine and AA metabolism correlate with STAT3 transcriptional activity in primary breast cancers.

Gene set enrichment analysis was performed on the GSE5460 breast cancer microarray dataset. 129 breast cancer samples were sorted according to (A) CTH, (B) CDO1, (C) COX2 (PTGS2), and (D) 5-LOX (ALOX5) mRNA expression. The 30 samples with the highest and lowest mRNA expression for each gene were analyzed for expression of a STAT3 gene expression signature. Statistical significance is presented as normalized p-value and normalized enrichment score (NES).