Molecular Effects of Doxorubicin on Choline Metabolism in Breast Cancer

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Abstract

Abnormal choline phospholipid metabolism is a hallmark of cancer. The magnetic resonance spectroscopy (MRS) detected total choline (tCho) signal can serve as an early noninvasive imaging biomarker of chemotherapy response in breast cancer. We have quantified the individual components of the tCho signal, glycerophosphocholine (GPC), phosphocholine (PC) and free choline (Cho), before and after treatment with the commonly used chemotherapeutic drug doxorubicin in weakly metastatic human MCF7 and triple-negative human MDA-MB-231 breast cancer cells. While the tCho concentration did not change following doxorubicin treatment, GPC significantly increased and PC decreased. Of the two phosphatidylcholine-specific PLD enzymes, only PLD1, but not PLD2, mRNA was down-regulated by doxorubicin treatment. For the two reported genes encoding GPC phosphodiesterase, the mRNA of GDPD6, but not GDPD5, decreased following doxorubicin treatment. mRNA levels of choline kinase α (ChKα), which converts Cho to PC, were reduced following doxorubicin treatment. PLD1 and ChKα protein levels decreased following doxorubicin treatment in a concentration dependent manner. Treatment with the PLD1 specific inhibitor VU0155069 sensitized MCF7 and MDA-MB-231 breast cancer cells to doxorubicin-induced cytotoxicity. Low concentrations of 100 nM of doxorubicin increased MDA-MB-231 cell migration. GDPD6, but not PLD1 or ChKα, silencing by siRNA abolished doxorubicin-induced breast cancer cell migration. Doxorubicin induced GPC increase and PC decrease are caused by reductions in PLD1, GDPD6, and ChKα mRNA and protein expression. We have shown that silencing or inhibiting these genes/proteins can promote drug effectiveness and reduce adverse drug effects. Our findings emphasize the importance of detecting PC and GPC individually.

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Introduction

The choline containing metabolites phosphocholine (PC) and glycerophosphocholine (GPC) are associated with malignant transformation and have been proposed as biomarkers of tumor progression [1]. PC, GPC, and free choline (Cho) can be detected by 1H and 31P MRS in vivo [1,2], as well as ex vivo using magic angle spinning MRS of biopsied tissue [3,4] or extracts of tissues or cells [5]. 1H or 31P MRS detected changes in PC and/or GPC can also serve as biomarkers for developing targeted anti-cancer drugs, such as drugs targeting choline kinase α [6,7], fatty acid synthase [8], or HSP90 [9], among others. Proton MRS of PC and the unresolved total choline (tCho) signal has also been applied to monitoring breast tumor response to therapy during radio- and chemotherapy in breast cancer patients [10–12]. As chemotherapeutic drugs are often toxic and different patients respond differently to the same dose of the same drug [13,14], developing biomarkers for monitoring the clinical response to therapy will help guide treatment choices, dosage, and personalized medicine.

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timing to achieve optimized therapeutic results with minimal side effects. Many clinical 
$^{3}$H and $^{31}$P MRS studies have reported changes in PC, GPC, and tCho following chemotherapy in human cancers, showing the promise of using these metabolic changes as an indicator of treatment response [10,15,16]. However, mechanistic molecular studies of how exactly individual chemotherapeutic drugs change the choline metabolite profile have not yet been performed in detail and would be important for the clinical interpretation of these noninvasive choline containing biomarkers.

Doxorubicin is a powerful first line chemotherapeutic drug widely used for cancer treatment, which unfortunately also possesses significant cardiotoxicity [17]. Doxorubicin cytotoxicity is caused by Topoisomerase II-mediated DNA damage, which has been reported as the major mechanism by which doxorubicin achieves cancer cell kill in the clinic. The same molecular mechanism of doxorubicin action has been shown to induce its cardiotoxicity, which limits the possibilities of alleviating doxorubicin’s main side effect and its application [18]. As doxorubicin induced heart failure is strongly dosage-dependent, it is of utmost importance to carefully plan clinical dosing regimens. This led us to evaluate the noninvasive biomarkers PC and GPC for possible longitudinal monitoring of doxorubicin treatment response for help with planning doxorubicin dosage and timing of its administration.

Choline containing metabolites in the cytosol are intermediates of choline phospholipid metabolism, which leads to de novo synthesis of the major cell membrane component phosphatidylcholine (PtdCho). Cancers and cancer cell lines display an activated choline metabolism, resulting in elevated cellular tCho and PC levels as a hallmark of cancer [1]. An elevated PC/GPC ratio is associated with tumor malignancy in breast and ovarian cancer cells [5,19,20]. PtdCho synthesis is catalyzed by several enzymes, whose expression and activity are regulated by oncogenic signaling pathways [1]. In cancer cells, free choline is released from the membrane by PtdCho specific phospholipase D enzymes, which are encoded by two genes: PLD1 and PLD2 [1]. Both genes are involved in cell proliferation, cell migration, cell survival, neoplastic transformation, and tumor progression [21], which makes them potential therapeutic targets [22]. Choline Kinase α (ChKα), which phosphorylates free choline to form PC, was reported to be up-regulated in a variety of cancer cell lines and tumor biopsy samples, and its deregulation was proposed as one of the main molecular causes of altered cellular PC levels [1,5,6,20,23–25]. Silencing or inhibition of ChKα in cancers decreases cell proliferation and reduces tumor xenograft growth [24–26]. Silencing of ChKα with lentivirally delivered shRNA or inhibiting ChKα enzyme activity with chemical compounds in tumor xenografts also significantly decreased tumor PC and tCho levels, which can be detected by single-voxel $^{1}$H and $^{31}$P MRS or $^{1}$H MRSI, suggesting that ChKα might be an anti-cancer target of theranostic value [23,26]. Enzymes with glycerophosphocholine phosphodiesterase (GPC-PDE) activity, which convert GPC to glycerol phosphate and free choline, deprive the cellular GPC pool and increase the PC/GPC ratio. Recently, two enzymes with GPC-PDE activity have been identified, which are glycerophosphodiester phosphodiesterase domain containing 5 (GDPD5), which osmotically regulates GPC levels in kidney cells [27], and GDPD6 (also named EDI3), whose knockdown decreased cancer cell motility [28]. Elevated GDPD5 expression is associated with breast cancer malignancy as well as with an increased PC and tCho content and elevated PC/GPC [29]. GDPD5 siRNA treatment of MCF7 cells decreased cell viability/proliferation and migration as compared to non-target siRNA [30]. Comparing GDPD5 and GDPD6 siRNA treatment of the breast cancer cell lines MCF7 and MDA-MB-231 suggested that GDPD6 takes a leading role in cleaving cellular GPC, while both GDPD5 and GDPD6 silencing decreased breast cancer cell migration and invasion [30]. It is possible that choline metabolic enzymes play a role in conferring the efficacy of chemotherapeutic drugs, which in turn may lead to changes in the noninvasive treatment response biomarkers PC, GPC, and tCho. These enzymes may also serve as potential therapeutic targets that can enhance the cytotoxicity of chemotherapeutic drugs.

In this study, we report changes in the choline metabolite profile following doxorubicin treatment in breast cancer cells, and explore the possibility of combining doxorubicin treatment with targeting the choline phospholipid pathway. We have observed that after doxorubicin treatment, although the tCho level did not change significantly, cellular GPC level increased and PC level decreased, leading to a significant PC/GPC ratio decrease. These metabolic changes were caused by PLD1, ChKα, and GDPD6, all of which were down-regulated at the mRNA and protein level after doxorubicin exposure. Silencing of these genes or inhibition of the enzyme activity of PLD1 potentiated low dose doxorubicin effects. Silencing of GDPD6 counteracted the detrimental effects of increased cancer cell migration ability, which was promoted by low concentrations of doxorubicin.

Materials and Methods

Cell Lines, Cell Culture, and Doxorubicin Treatment

The breast cancer cell lines MDA-MB-231 and MCF7 were originally obtained from ATCC (American Type Culture Collection, Manassas, VA) and maintained in RPMI-1640 (Sigma-Aldrich, St. Louis, MO) or MEM (Sigma-Aldrich) medium supplemented with 10% of fetal bovine serum (FBS), respectively. Cells were grown in an incubator at 37 °C with 5% CO$_2$ in a humidified atmosphere. Cell lines tested negative for mycoplasma using a PCR-based MycoAlert kit (Greiner Bio-One, Monroe, NC) and were authenticated by STR profiling, both of which is routinely done every six months. All experiments were performed between passage numbers 2–10 after cell thawing from the same frozen batch of cells with identical passage number. MCF7 and MDA-MB-231 cells were treated with 5 μM doxorubicin hydrochloride in standard cell culture medium for 24 h or 48 h, as recently described by us [31]. We chose a 5 μM concentration because in the clinic, doxorubicin is typically supplied as a bolus that results in plasma concentrations of 5 μM for MDA-MB-231 and 10 μM for MCF7 [32]. We have previously determined the IC$_{50}$ values for 24 h and 48 h of doxorubicin treatment in our cell culture systems to be 4.0 ± 0.3 μM and 2.7 ± 0.5 μM for MCF7 cells, respectively, and 4.0 ± 0.1 μM and 1.4 ± 0.2 μM for MDA-MB-231 cells, respectively [31]. We prepared all untreated vehicle controls at the same time from the same respective cell batch.

Metabolite Extraction and High-Resolution MRS

Cells were provided with fresh cell culture media and treated with 5 μM doxorubicin (Sigma-Aldrich), or DMSO as control, for 24 h or 48 h, followed by cell harvesting by trysinization, and washing with saline. Cell pellets were obtained by centrifugation, re-suspended, and cells were extracted with 4 ml of cold methanol, then 4 ml of saline. Cell pellets were obtained by centrifugation, re-suspended, and washing with saline. Cell pellets were obtained by centrifugation, re-suspended, and washing with saline. Cell pellets were obtained by centrifugation, re-suspended, and washing with saline. Cell pellets were obtained by centrifugation, re-suspended, and washing with saline.

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complete phase separation, and then the upper water phase was taken out, subjected to cation ion removal with chelax beads (Sigma-Aldrich), rotary distillation, and lyophilization. The dried metabolite samples were stored at −80 °C until measurement. Each metabolite sample was dissolved in deuterium dioxide containing 0.24×10^−6 mol 3-(trimethylsilyl)propionic-2,3,3-d4 acid (TSP, Sigma-Aldrich) as chemical shift reference and standard for metabolite quantification. Each lipid sample was dissolved in 0.6 ml of CDCl3/CD3OD (2:1, v/v) containing premixed 2.17×10^−6 mol tetramethylsilane as an internal concentration standard (Cambridge Isotope Laboratories, Inc.). Fully relaxed high-resolution 1H MR spectra were acquired on a Bruker Avance 500 MR Spectrometer (Bruker BioSpin Corp., Billerica, MA) as previously described [5]. Free choline (3.209 ppm), PC (3.227 ppm), N-(CH3)3 signal) from the lipid spectra were quantified by peak integration using MestReNova software (Mestrelab Research, Santiago de Compostela, Spain) and normalized to cell number as previously described [5]. Each experiment was repeated 3 times. Other metabolic changes were also quantified from the 1H spectra, as recently published by us [31].

RNA Extraction and qRT-PCR

Cells were treated with 5 μM doxorubicin for 24 h or 48 h prior to harvesting the sample. RNA was extracted using the RNAsesy mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. RNA concentration was measured using a spectrophotometer at its absorption maximum of 260 nm. cDNA was synthesized with qScript cDNA supermix (Quanta Bioscience, Gaithersburg, MD) according to the manufacturer’s protocol. Real-time quantitative PCR mixture was set up with SYBR green supermix (Quantabioscience), and thermal cycling was run on a Biorad CFX384™ system (Bio-Rad Laboratories, Hercules, CA). The results were processed using the 2−ΔΔCT method [26]. The primers used for qRT-PCR were as follows. PLD1 forward: 5′-GGAAGGCGTGCAGGTGAATG-3′. PLD1 reverse: 5′-GATAGCCCGAGAAGACAACCCTAAA-3′. PLD2 forward: 5′-GCCAGCACCTCACCTACATT-3′. PLD2 reverse: 5′-TCTCTGACAGTCCACCTACT-3′. ChkX forward: 5′-GATCCGAACAGCTGAAAGAAATG-3′. ChkX reverse: 5′-CGGCTCGGGATGAGTCTGC-3′. GDPD5 forward: 5′-CTA CAACCCCTGACGAT-3′. GDPD5 reverse: 5′-AACATAGGAG AGCAGAT-3′. GDPD6 forward: 5′-TTTCAAAATGCTGCAGGGT AAT-3′. GDPD6 reverse: 5′-ACCCAAAAGAGCAACAGTGTGA-3′. 36B4 forward: 5′-GATTGCGTACCCCAACTGTTGCA-3′. 36B4 reverse: 5′-CAGGGCAGCAGCACAAGGC-3′. Each experiment was performed with two technical replicates, and three biological repeats.

Western Blotting

After treatment with different concentrations of doxorubicin (0.0, 0.5, 1.5, 5.0 μM) for 24 h, cells were gently washed with PBS (Mediatech, Manassas, VA) then RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% Triton X100, 0.1% SDS) supplemented with protease inhibitor mixture (P8340, Sigma-Aldrich) was added. Cells in RIPA buffer were scraped into a vial and broken by sonicking them 10 times with a 1 second pulse and 1 second stop each with samples bathing in ice. After centrifugation, the supernatant was mixed with 6X SDS-PAGE loading buffer (500 mM Tris–HCl, pH 6.8, 600 mM DTT, 12% SDS, 0.6% bromophenol blue, 60% glycerol), and soluble proteins were completely denatured by heating at 90 °C for 5 min. Equal amounts of total proteins were loaded and separated by PAGE, and transferred to PVDF membranes. After blocking with 5% non-fat dry milk for 1 h, membranes with bands of interest were incubated with primary antibody overnight while shaking in the cold room at 4 °C. Primary antibodies were mouse anti-PLD1 antibody (Santa Cruz Biotechnology, Dallas, TX) diluted 1:100, or custom-made rabbit anti-ChkX antibody [24] diluted 1:250. Membranes were washed three times with PBS plus 0.1% Tween 20 (PBST), followed by incubation for 1 h with secondary horseradish peroxidase conjugated anti-mouse or anti-rabbit antibody diluted 1:3000 and another three PBST washes. Signal was developed with SuperSignal West Pico chemiluminescent substrate kit (Thermo Fisher Scientific, Waltham, MA) and its intensity was detected with classic X-ray film (Research Products International, Mt. Prospect, IL) in a dark room. Films were scanned and immunoreactive bands were quantified by densitometry using Image J (NIH, Bethesda, MD) as previously described [24]. Each experiment was repeated 3 times.

siRNA Transfection

Stock solutions of 100 μM siRNA in RNase free water were prepared and transfected with lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer’s protocol to give a final concentration of 60 nM. Cells were incubated with siRNA:lipofectamine 2000 complex for 48 h prior to performing cell migration or cell viability assays. The knockdown efficiency of all siRNA sequences used in this study was confirmed in previous studies. The following siRNA sequences were used: The PLD1 siRNA sequence was 5′-GGGAAGGAGGAGCACAGAA-3′ [22], the ChkX siRNA sequence was 5′-CATGCTGTTCCAGTGCTCC-3′ [26], the GDPD6 siRNA sequence was 5′-GAGAAGTCTCCTGTTGCTCA-3′ [30], and the non-target siRNA sequence was 5′-GTGGAACCTTGTAAATCT-3′.

Cell Viability Assay

Cells following 48 h of siRNA transfection or intact cells were seeded in a 96-well plate at 5000 cells per well. After the cells attached to the bottom, a series of doxorubicin concentrations was applied to the wells. For PLD1 inhibitor experiments, a series of doxorubicin concentrations combined with 10 μM of PLD1 selective inhibitor VU0155069 (Santa Cruz Biotechnology) was applied to the wells after 48 h of seeding intact cells. After 48 h or 72 h of treatment, WST-1 assay reagent (Roche Life Science, Indianapolis, IN) was added according to the manufacturer’s protocol and incubated for 2 h, followed by detecting the absorption at 450 nm. We measured 3 technical replicates for each of 4 biological repeats for each experimental group.

Cell Migration Assay

MDA-MB-231 cells incubated with siRNA:lipofectamine 2000 for 48 h were trypsinized and washed with RPMI-1640 medium without serum. A 24-well plate was loaded with RPMI-1640 medium with 2% FBS, and 100 nM of doxorubicin was added as treatment or DMSO as control. Each Corning transwell insert with 8 μm pore (product number 3422, Corning, Tewksbury, MA) was loaded with RPMI-1640 medium without serum plus 100 nM doxorubicin as treatment or DMSO as control and 8000 cells. After 24 h, the cells in the top of inserts were removed by cotton swabs; the cells attached to the bottom of inserts were fixed with 4% paraformaldehyde (PFA,
Santa Cruz Biotechnology) and stained with 1% crystal violet solution. Representative views of transwell insert membranes were imaged with a Nikon TS100 inverted microscope equipped with a charge-coupled device (CCD) camera. Cell numbers in four representative fields of view per transwell insert membrane were counted manually. Each experiment was repeated 3 times.

**Statistical Analysis**

All data were processed in Microsoft Office Excel (Microsoft, Redmond, WA). Comparisons were made using unpaired two-tailed Student’s t test and considered significant if the P values were ≤0.05. Each experiment was repeated at least three times as indicated at the end of each experimental subsection. Data are presented as mean ± standard error (SE).

**Results**

**Choline Metabolite Profile Changes in Doxorubicin-Treated Breast Cancer Cells**

High-resolution 1H MR spectra of the water-soluble metabolites from estrogen receptor (ER) positive MCF7 and triple negative MDA-MB-231 breast cancer cell extracts demonstrated significant GPC increases and PC decreases upon treatment with 5 μM doxorubicin for 24 h, which were even more pronounced after 48 h of treatment in both cell lines compared to the respective controls (Figure 1A). Quantification of each choline metabolite showed that after 24 h of doxorubicin exposure, the GPC level increased significantly in both MCF7 (P = .011), and MDA-MB-231 (P = .028) cells, while the PC level decreased slightly, thus leading to an increased PC/GPC ratio in MCF7 (P = .028) and MDA-MB-231 (P = .038) cells (Figure 1B). After 48 h of doxorubicin treatment, the GPC level further increased. In MCF7, the GPC concentration of doxorubicin treated samples was about 2.5 times that of untreated samples (P = .009), and in MDA-MB-231, it was increased by about 3.5 times (P = .020). At the same time, the PC concentration decreased significantly after 48 h of doxorubicin treatment in both breast cancer cell lines, leading to levels of about 65% of control in MCF7 (P = .006) and about 54% of control in MDA-MB-231 (P = .002) cells. After 48 h of doxorubicin exposure, PC/GPC ratios significantly decreased to less than 1.0 in both MCF7 (P = .005) and MDA-MB-231 (P = .005) cells (Figure 1C). Free choline levels were negligible compared to GPC and PC in both cell lines, and differences between doxorubicin treated cells and control cells were not significant in any case. The tCho level, which is the sum of PC, GPC, and Cho, did not change following doxorubicin treatment in either cell line at any treatment time point in spite of the significant increases in GPC and decreases in PC. PtdCho levels significantly increased following doxorubicin treatment for 48 h in MCF7 (P = .036), and for 24 h (P = .034) and 48 h (P = .033) of treatment in MDA-MB-231 breast cancer cells. Other metabolic changes quantified from the 1H MR spectra were recently published by us, and comprised consistent increases in creatine and phosphocreatine in both cell lines in addition to the GPC elevation [31].

**Doxorubicin Down-regulates Selected Choline Metabolic Enzymes**

Based on the significant doxorubicin-induced changes in GPC and PC, we explored the molecular mechanisms underlying these changes by qRT-PCR screening of genes encoding important enzymes in PtdCho metabolism that potentially regulate cellular GPC and PC levels. Of the two tested GPC-PDE genes, only mRNA of GDPD6 was decreased consistently in both MCF7 cells at 24 h (decrease by 75%, P = .001) and MDA-MB-231 cells at 24 h (decrease by 52%, P = .006) and 48 h (decrease by 70%, P = .030) compared to control (Figure 2A). Gene expression levels for MCF7 cells were only obtained at 24 h of doxorubicin treatment, as it was not possible to obtain high quality RNA at 48 h of treatment in MCF7 due to severe cell death. The GDPD5 gene only showed a significant decrease in MDA-MB-231 cells at 24 h of doxorubicin exposure (decrease by 58%, P = .010) (Figure 2A). Therefore, with both GDPD5 and GDPD6 reported to confer GPC-PDE activity, our data show that GDPD6 mostly contributed to the doxorubicin induced GPC increase in breast cancer cells. The ChKα gene was also down-regulated in MCF7 cells at 24 h (decrease by 64%, P = .049) and MDA-MB-231 cells at 24 h (decrease by 90%, P < .001) and 48 h (decrease by 80%, P = .008) after doxorubicin treatment of breast cancer cells as compared to the respective vehicle controls, which was consistent with the decrease in PC levels following doxorubicin treatment (Figure 2C). In addition, PLD1 expression was decreased upon doxorubicin treatment in both breast cancer cell lines, resulting in a decrease by 74% in MCF7 cells at 24 h (P = .021), by 84% in MDA-MB-231 cells at 24 h (P < .001) and by 74% at 48 h (P = .006) (Figure 2D). PLD2 gene expression remained unchanged, or increased by 81% in MDA-MB-231 cells following 48 h (P = .032) of doxorubicin exposure as compared to the respective vehicle controls (Figure 2E).

**Doxorubicin at Lethal and Sub-lethal Concentrations Decreases PLD1 and ChKα Protein Levels**

After identifying doxorubicin-induced changes in mRNA levels of choline metabolic enzymes, we further explored the relationship between down-regulation of the encoded proteins and doxorubicin concentration used for treatment. At the lethal concentration of 5 μM of doxorubicin, both PLD1 (Figure 3A) and ChKα (Figure 3B) protein levels significantly decreased compared to vehicle treated control cells. In MCF7 cells, PLD1 protein decreased to 40% of control (P = .035) and ChKα to 20% of control (P = .022). In MDA-MB-231 cells, PLD1 was reduced to 30% (P = .041) and ChKα to 17% (P = .005) of control. At a sub-lethal concentration of 1.5 μM of doxorubicin, PLD1 significantly decreased (P = .017) while ChKα marginally decreased as compared to vehicle controls in MCF7 cells. In MDA-MB-231 cells at 1.5 μM doxorubicin, PLD1 and ChKα were also slightly reduced as compared to control. At a non-lethal concentration of 0.5 μM of doxorubicin, neither PLD1 nor ChKα protein levels were changed. In conclusion, doxorubicin treatment resulted in a reduction of PLD1 and ChKα protein levels that was more pronounced at higher concentrations of doxorubicin.

**Disrupting PtdCho Metabolism Potentiates Doxorubicin Toxicity**

Based on the discovery that doxorubicin reduces PLD1 and ChKα protein expression levels, we next tested if silencing or inhibiting PLD1, ChKα, or GDPD6 could potentiate the effects of lower doxorubicin concentrations. Treating PLD1-silenced cells with doxorubicin resulted in significantly decreased cell viability as compared to that of non-target siRNA treated control cells at a concentration of 500 nM (P = .025) and 1500 nM (P = .019) doxorubicin in MDA-MB-231 cells, whereas no significant effects in
Figure 1. Doxorubicin treatment induced changes in choline containing metabolite profiles of breast cancer cell lines. (A) Representative $^1$H MR spectra of the choline metabolite region of the water-soluble phases of MCF7 (left) and MDA-MB-231 (right) cell extracts obtained following 24 h or 48 h of 5 $\mu$M doxorubicin treatment (treated) or vehicle (DMSO) control (control). In both cell lines, doxorubicin treatment elevated GPC while PC decreased, which was more pronounced at 48 h of treatment than at 24 h. (B, C) Quantification of choline-containing metabolite concentrations showed that the total choline containing metabolites (tCho) concentration did not change between treated and control samples in MCF7 (left) and MDA-MB-231 (right) cells, while GPC increased, PC decreased, thus decreasing the PC/GPC ratio dramatically following (B) 24 h and (C) 48 h of 5 $\mu$M doxorubicin treatment compared to the respective vehicle controls. (D) Quantification of PtdCho N-(CH$_3$)$_3$ signal in lipid spectra from MCF-7 (left) and MDA-MB-231 (right) cell extracts obtained following 24 h or 48 h of 5 $\mu$M doxorubicin treatment (treated) or vehicle (DMSO) control (control). All quantification results are from three separate experiments and expressed as mean + SE for metabolite spectra in B and C, and mean + SD for lipid spectra in D. *$P \leq .05$, **$P \leq .01$. An unpaired two-tailed $t$ test was used for all comparisons.
MCF7 cells (Figure 4A, B). ChKα silencing combined with low dose doxorubicin treatment did not show any effect in MDA-MB-231 cells, but significantly reduced cell viability of ChKα-silenced MCF7 cells treated with 1500 nM doxorubicin ($P < .001$). While previous studies showed that silencing of ChKα was able to significantly reduce cell viability in breast cancer cells at an siRNA concentration of approximately 100 nM for 48 h [24], here we have chosen a lower siRNA concentration of 60 nM to be able to observe additional reductions in cell viability caused by the combination with doxorubicin treatment. GDPD6 silencing combined with low dose doxorubicin treatment reduced cell viability in both breast cancer cell lines. In MDA-MB-231 cells, the viability of 500 nM ($P = .019$) and 1500 nM ($P = .014$) doxorubicin treated GDPD6-silenced cells significantly decreased compared to that of non-target siRNA treated control cells. In MCF7 cells, the combined treatment effect was slight at 500 nM doxorubicin ($P = .082$), and highly significant at 1500 nM doxorubicin ($P < .001$). We also observed that the PLD1 specific inhibitor VU0155069 [33] acted synergistically with doxorubicin in MCF7 cells (Figure 4C). Although VU0155069 alone had no effect on MCF7 cells, it dramatically exacerbated the effect of doxorubicin on cell viability in the concentration range above 150 nM (Figure 4C). VU0155069 acted additively when combined with doxorubicin in decreasing the cell viability of MDA-MB-231 cells (Figure 4D).

**GDPD6, but not PLD1 and ChKα, Silencing Counteracts Doxorubicin Induced Cell Migration**

Previous reports have shown that low concentrations of doxorubicin were able to promote breast cancer cell migration [34], while there is evidence that both PLD1 [35] and GDPD6 [28,30] silencing can decrease cancer cell migration. To test if silencing of these genes is able to inhibit this undesired effect of doxorubicin on breast cancer cell migration, we performed transwell migration assays under combination treatment. Our observations confirmed that 100 nM of doxorubicin was able to double the number of migrated cells ($P = .006$). Silencing alone of PLD1 ($P = .009$) or GDPD6 ($P = .036$) significantly decreased MDA-MB-231 breast cancer cell migration as compared to non-target siRNA control without doxorubicin treatment. Silencing of PLD1 was not able to reduce doxorubicin-promoted cell migration. When 100 nM doxorubicin was added to PLD1-silenced MDA-MB-231 cells, the migrated cell number was almost the same as that of non-target siRNA treated cells without doxorubicin. In contrast, GDPD6 silencing was able to counteract low dose doxorubicin-induced cell migration (Figure 5), as evident from a significantly reduced number of migrated cells in 100 nM doxorubicin treated GDPD6-silenced MDA-MB-231 cells as compared to non-target siRNA treated cells under low dose doxorubicin treatment ($P < .001$). Silencing of ChKα did not result in significant changes in migration compared to non-target siRNA control without doxorubicin treatment, and was not able to mitigate the doxorubicin-induced increase in MDA-MB-231 cell migration.

**Discussion**

In this study, we demonstrated that the chemotherapeutic drug doxorubicin significantly increased GPC and decreased PC in human breast cancer cells through down-regulation of the PtdCho metabolism enzymes PLD1, ChKα, and GDPD6, which was evident at the mRNA and protein levels, and which occurred in a doxorubicin concentration dependent manner. Targeting these genes by siRNA silencing or inhibition in combination with doxorubicin treatment further reduced the cell viability of the treated breast cancer cells. Silencing of GDPD6 in combination with low dose doxorubicin treatment was able to counteract doxorubicin-induced cell migration.
Following doxorubicin treatment of breast cancer cells, we observed that the tCho signal did not change, while its major component PC decreased and GPC increased, resulting in a significantly smaller PC/GPC ratio than that in untreated cells. Alterations in choline metabolite concentrations have been reported in response to the chemotherapeutic drug docetaxol in cell and xenograft models, demonstrating a significant decrease in intracellular PC and increase in GPC following docetaxol treatment [36], which is consistent with our observations in doxorubicin-treated human breast cancer cells. These observations are also consistent with the finding that high GPC and low PC are typical of a less malignant phenotype in breast and ovarian cancer cells [19,37]. Previous clinical in vivo studies in breast cancer patients have monitored alterations in the tCho signal in response to chemotherapeutic drug treatments [10–12,38, 15], but MRS based tCho measurements may be unable to distinguish response earlier than MR imaging based size measurements [12,15,38]. Quantitative tCho measurements from in vivo MRS data in a multi-site clinical trial setting of neoadjuvant chemotherapy in breast cancer patients were hampered by the technical difficulties of acquiring these data consistently at multiple sites [39]. Most of these patient studies used a combination of different chemotherapeutics, some of which included doxorubicin [10–12,38]. For example, Baek et al. [15] have studied breast cancer patients receiving neoadjuvant combination therapy with doxorubicin-cyclophosphamide and observed a trend of tCho decrease in responding patients, providing some patient-based evidence that links doxorubicin treatment with choline metabolism. In addition, our study suggests that an unchanged tCho peak following treatment may be misleading under certain circumstances, and that detailed studies are necessary to address how individual metabolites including GPC and PC respond to each individual chemotherapeutic drug in cell and animal model. The ability to detect separated PC and GPC signals in vivo is a challenging but worthwhile goal considering the fact that they may behave differently in response to the same treatment. Significant recent advances in detecting separated PC and GPC signals along with the ethanolamine counterparts phosphoethanolamine and glycerophosphoethanolamine in patients in vivo have been reported with specialized in vivo 31P MRS techniques [40] and at high fields such as 7 T [2]. Moreover, high-resolution 1H MRS on biopsy specimens from cancer patients is a powerful approach that is able to resolve the tCho signal into GPC, PC, and Cho [3,41–43]. Both of these approaches could be used for detecting treatment-induced changes that increase GPC while concomitantly decreasing PC, leaving net tCho unchanged in patients in vivo or in biopsies from cancer patients.

The use of noninvasive metabolic imaging biomarkers of tumor response would be particularly helpful for detecting the response of a given tumor early on during the initially selected treatment regimen. It is well known that anatomical MRI measures of tumor size according to the “Response Evaluation Criteria In Solid Tumors” (RECIST) may require up to three cycles of chemotherapy for a response evaluation, which is rather slow [44]. The biomarkers PC and GPC as detected noninvasively by 31P MRS would enable a response evaluation as early as 24 h following the start of the treatment. Such an early detection of tumor response would be helpful for choosing the right drug, as well as adjusting its timing of administration and dosage during treatment. Detection of PC and GPC could be combined with other information obtained from MRI such as pharmacokinetic parameters, apparent diffusion coefficient, T2 relaxation time, and water-to-fat ratio, which have been reported as biomarkers of anticancer therapy [15]. The advantage of detecting PC and GPC concentration for cancer treatment response evaluation would be that these biomarkers provide information about distinct molecular pathways.

The three enzymes PLD1, ChKα, and GDPD6 were down-regulated by doxorubicin treatment in breast cancer cells. An altered expression of ChKα resulting in PC and sometimes tCho level changes can be observed in response to several targeted therapeutic agents including inhibitors of RAS [45], PI3K [6], PI-PLC [46], fatty acid synthase [8], Heat Shock Protein 90 [9], and histone deacetylase.

| Figure 3. | ChKα and PLD1 protein expression levels in MCF7 and MDA-MB-231 breast cancer cells decreased in a doxorubicin concentration dependent manner. (A) ChKα and (B) PLD1 protein levels decreased with increasing doxorubicin concentrations in a concentration dependent manner in MCF7 (left) and MDA-MB-231 (right) breast cancer cells, and both proteins were significantly decreased at lethal concentrations of 1.5 and 5.0 μM doxorubicin. *P ≤ .05, **P ≤ .01. An unpaired two-tailed t test was used for all comparisons. |
but effects of chemotherapeutic drugs on the regulation of ChKα are relatively unknown. We provided for the first time evidence that the chemotherapeutic drug doxorubicin down-regulated ChKα and decreased cellular PC levels in breast cancer cells. To the best of our knowledge, there are no reports about any drug effects on GDPD6 expression. We are reporting here that doxorubicin decreased GDPD6 expression and in turn increased the cellular GPC concentration in breast cancer cells. The doxorubicin concentration was linked to the level of protein expression in our study, resulting in a more pronounced reduction of PLD1 and ChKα protein levels at higher doxorubicin concentrations. Overall, our data suggest the possibility that GDPD6, PLD1, and ChKα protein reduction, and the resulting GPC increase and PC decrease, were associated with doxorubicin toxicity in the treated breast cancer cells. This combined reduction of counteracting enzyme expression levels most likely also resulted in unaltered Cho levels, as the reduction in PLD and GDPD6 levels would both reduce Cho levels, and the reduction in ChKα would increase Cho levels at the same time, leading in sum to no significant Cho change. PLD1 knockdown has previously been reported to increase PC level as it reversely interacts with ChKα expression [22]. In our study, these reversely interacting effects in the expression levels of PLD1 and ChKα were most likely masked by a direct down-regulation of ChKα expression by doxorubicin. The significant increase in membrane PtdCho levels following doxorubicin treatment could result from reduced breakdown of PtdCho due to reduced PLD expression levels upon doxorubicin treatment. Gene expression analysis of enzymes in choline phospholipid metabolism following drug treatment may help undermine the MRS-detected choline metabolite level profile that can serve as biomarker profile of treatment response.

Based on our observations, we further tested the possibility of targeting PtdCho metabolism in combination with doxorubicin treatment to enhance cell kill or exacerbate the reduction in cancer cell proliferation. PLD1 silencing or inhibiting PLD1 enzyme activity enhanced the doxorubicin-mediated reduction in cell viability in an additive or synergistic way depending on the cell line. The triple-negative breast cancer cell line MDA-MB-231 with high basal levels of PLD1 expression [22] had a more sensitive response to
PLD1 knockdown or inhibition than the estrogen receptor positive cell line MCF7, which is consistent with reports demonstrating that MDA-MB-231 cells rely on PLD activity for survival signals [21]. ChKα was previously reported to show synergistic effects in combination with 5-fluorouracil in decreasing cell viability in breast cancer cells [48]. Combining ChKα inhibitors with 5-fluorouracil was demonstrated as an effective way to treat colorectal cancer in preclinical models [49]. There are several ChKα inhibitors available [50,51]. Effective ways to include ChKα inhibitors into chemotherapy treatment regimes in combination with doxorubicin will need more detailed investigations. Studies on GDPD6 in breast cancer are relative limited as of yet. Our study has shown quite promising results for combining GDPD6 with doxorubicin as GDPD6 silencing potentiated doxorubicin induced cell kill in both breast cancer cell lines tested. GDPD6 as a relatively new anticancer target requires a lot more investigation into the molecular pathways it is connected with in cancer. Development of GDPD6 inhibitors may be a worthwhile undertaking for further testing GDPD6 as anticancer treatment target.

We demonstrated that GDPD6 knockdown was able to disrupt doxorubicin promoted cell migration, which occurs at sub-lethal doses of doxorubicin. Several types of cytotoxic therapeutic drugs, including doxorubicin, were reported to have adverse tumor-promoting effects, which may lead to chemotherapy-driven metastasis that puts patients’ lives at risk [52]. This effect has been proposed to occur through tumor-protective immune responses, but doxorubicin itself has also been reported to increase cell migration in vitro, suggesting that the drug itself can aggravate cancer cell mobility [34]. Our study confirmed the ability of doxorubicin to enhance MDA-MB-231 breast cancer cell migration at sub-lethal doses of 100 nM. A reasonable choice of combination therapy, which includes additional targeted therapeutic agents to counteract the
adverse effects of chemotherapeutic drugs, is necessary. Although PLD1 knockdown [28] and PLD1 inhibition by small molecules [33] was shown to decrease cell migration in MDA-MB-231 cells, and ChKα knockdown was reported to decrease ovarian cancer cell migration and aggressiveness [53], in our study, only the knockdown of GDPD6 was able to counteract doxorubicin promoted cell migration. Careful examination of the interaction between chemo-therapeutic drugs and targeted therapy agents may lead to new treatment regimens, which eliminate primary tumors more effectively while reducing the risk of possible chemotherapy-driven metastasis in the clinic.

In summary, our study demonstrated for the first time that the chemotherapeutic drug doxorubicin resulted in decreased ChKα, PLD1, and GDPD6 expression levels, which resulted in elevated GPC and reduced PC in human breast cancer cells. These findings provide the basis for noninvasive biomarker development of treatment response based on choline phospholipid metabolism. We also showed that doxorubicin combines well with silencing or inhibiting ChKα, PLD1, and GDPD6 to enhance breast cancer cell kill. The effects of doxorubicin on reducing ChKα and PLD1 protein levels suggest an important role of these proteins in the cell kill mechanism of doxorubicin. In particular the finding that GDPD6 silencing was able to counteract low dose doxorubicin-mediated cell migration of triple-negative breast cancer cells makes combining GDPD6 targeting with doxorubicin a hopeful choice to further explore as a potential treatment regimen for breast cancer, which could be monitored directly by suitable noninvasive MRS approaches. Future studies to investigate the metabolic profiles of cancer cells treated with different choline-modifying enzyme inhibitors and/or siRNAs in combination with doxorubicin will be able to further corroborate existing links between enzymatic changes and resulting metabolic findings, thereby identifying biomarkers for these combined therapeutic approaches.

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References
[1] Wijnen JP, Jiang L, Greenwood TR, Cheng M, Döpkens M, Cao MD, Bhujwalla ZM, Krishnamachary B, Klomp DWJ, and Gunde K (2014). Silencing of the glycerophosphocholine phosphodiesterase GDPD5 alters the phospholipid metabolite profile in a breast cancer model in vivo as monitored by 31P MRS. NMR Biomed 27, 692–699.
[2] Wijnen JP, van der Kemp WJM, Lurtje MP, Korteweg MA, Luijten PR, and Klomp DWJ (2012). Quantitative 31P magnetic resonance spectroscopy of the human breast at 7 T. Magn Reson Med 68, 339–348.
[3] Cao MD, Sitter B, Bathen TF, Bofin A, Lønning PE, Lundgren S, and Gunde K (2012). Predicting long-term survival and treatment response in breast cancer patients receiving neoadjuvant chemotherapy by MR metabolic profiling. NMR Biomed 25, 360–378.
[4] Sitter B, Sonnewald U, Spraul M, Fjosne HE, and Gribbestad IS (2002). High-resolution magic angle spinning MRS of breast cancer tissue. NMR Biomed 15, 327–337.
[5] Glunde K, Jue C, and Bhujwalla ZM (2004). Molecular Causes of the Aberrant Choline Phospholipid Metabolism in Breast Cancer. Cancer Res 64, 4270–4276.
[6] Al-Saffar NMS, Jackson LE, Rainaud FI, Clarke PA, Ramirez de Molina A, Lacal JC, Workman P, and Leach MO (2010). The Phosphoinositide 3-Kinase Inhibitor PI-103 Downregulates Choline Kinase Leading to Phosphocholine and Total Choline Decrease Detected by Magnetic Resonance Spectroscopy. Cancer Res 70, 5507–5517.
[7] Beloueche-Babari M, Arunan V, Troy H, te Poele RH, Te Fong A-CW, Jackson LE, Payne GS, Griffthrs JR, Judson IR, and Workman P, et al (2012). Histone Deacetylase Inhibition Increases Levels of Choline Kinase and Phosphocholine Facilitating Noninvasive Imaging in Human Cancers. Cancer Res 72, 990–1000.
[8] Ross J, Najjar AM, Sankaranarayananpillai M, Tong WP, Kaiharuchi A, and Ronen SM (2008). Fatty acid synthase inhibition results in a magnetic resonance detectable drop in phosphocholine. Cancer Res 77, 2556–2565.
[9] Chung Y-L, Troy H, Banerji U, Jackson LE, Walton MI, Stubbs M, Griffthrs JR, Judson IR, Leach MO, and Workman P, et al (2003). Magnetic Resonance Spectroscopic Pharmacodynamic Markers of the Heat Shock Protein 90 Inhibitor 17-Allylamino,17-Demethoxygaladanamycin (17AAG) in Human Colon Cancer Models. J Natl Cancer Inst (Bethesda) 95, 1624–1633.
[10] Meisamy S, Bolan PJ, Baker EH, Bliss RL, Gubbalke E, Everson LI, Nelson MT, Emory TH, Tuttle TM, and Yee D, et al (2004). Neoadjuvant chemotherapy of locally advanced breast cancer: predicting response with in vivo 1H MR spectroscopy—a pilot study at 4 T. Radiology 233, 424–431.
[11] Payne GS and Leach MO (2006). Applications of magnetic resonance spectroscopy in radiotherapy treatment planning. Br J Radiol 79, S16–S26.
[12] Danisah KD, Sharma U, Sah RG, Seenu V, Parshad R, and Jagannath NR (2010). Assessment of therapeutic response of locally advanced breast cancer (LABC) patients undergoing neoadjuvant chemotherapy (NACT) monitored using sequential magnetic resonance imaging (MSI). NMR Biomed 23, 233–241.
[13] Mau M, Guarneri V, Frassolari A, and Conte PF (2006). Primary systemic therapy in operable breast cancer: clinical data and biological fall-out. Ann Oncol 17(Suppl 5), v158–v164.
[14] Fisher B, Bryant J, Wolmark N, Mamounas E, Brown A, Fisher ER, Wickerham DL, Bogovic M, DeCillis A, and Rebioud A, et al (1998). Effect of preoperative chemotherapy on the outcome of women with operable breast cancer. J Clin Oncol 16, 2672–2685.
[15] Hyeon-Man B, Jeon-Hor C, Ke N, Hon JY, Shadfar B, Rita SM, Orhan N, and Min-Ying S (2009). Predicting Pathologic Response to Neoadjuvant Chemotherapy in Breast Cancer by Using MR Imaging and Quantitative 1H MR Spectroscopy. Radiology 251, 653–662.
[16] Semmler W, Gadenmann G, Bachert-Baumann P, Zabel HJ, Lorenz WJ, and Gv (1998). Monitoring human tumor response to therapy by means of 31P. Radiology 166, 533–539.
[17] Minotti G, Menna P, Salvatorelli E, Cairo G, and Gianni L (2004). Anthracyclines: Molecular Advances and Pharmacologic Developments in Antitumor Activity and Cardioxicity. Pharmacol Res 46, 185–229.
[18] Zhang S, Liu X, Bawa-Kalife T, Lu L-S, Lyu YL, Liu LF, and Yeh ETH (2012). Identification of the molecular basis of doxorubicin-induced cardioxicity. Nat Med 18, 1639–1642.
[19] Aboagye EO and Bhujwalla ZM (1999). Malignant Transformation Alters Membrane Choline Phospholipid Metabolism of Human Mammary Epithelial Cells. Cancer Res 59, 80–84.
[20] Iorio E, Ricci A, Bagnoli M, Pisana ME, Castellano G, Di Vito M, Venturini E, Glunde K, Bhujwalla ZM, and Mezzananza D, et al (2010). Activation of Phosphatidylincholine Cycle Enzymes in Human Epithelial Ovarian Cancer Cells. Cancer Res 70, 2126–2135.
[21] Foster DA and Xu L (2003). Phospholipase D in Cell Proliferation and Cancer! National Cancer Institute, and the institutional support from the Research Centers in Minority Institutions (RCMI) program of the NIH. Mol Cancer Res 1, 789–800.
[22] Gadia M, Mori N, Cao MD, Mironchik Y, Kakkad S, Gribbestad IS, Glunde K, Krishnamachary B, and Bhujwalla ZM (2014). Phospholipase D1 and choline kinase-alpha are interactive targets in breast cancer. Cancer Biol Ther 15, 593–601.
[23] Al-Saffar NMS, Troy H, de Molina AR, Jackson LE, Madhu B, Griffthrs JR, Leach MO, Workman P, Lacal JC, and Judson IR, et al (2006). Noninvasive Magnetic Resonance Spectroscopic Pharmacodynamic Markers of the Choline Kinase Inhibitor MN58b in Human Carcinoma Models. Cancer Res 66, 427–434.
[24] Glunde K, Raman V, Mori N, and Bhujwalla ZM (2005). RNA interference-mediated choline kinase suppression in breast cancer cells induces differentiation and reduces proliferation. Cancer Res 65, 11034–11043.
[25] Ramirez de Molina A, Rodriguez-Gonzalez A, Gutierrez R, Martinez-Pineiro L, Sanchez J, Bonilla F, Rosell R, and Lacal J (2002). Overexpression of choline kinase is a frequent feature in human tumor-derived cell lines and in lung,
prostate, and colorectal human cancers. Biochem Biophys Res Commun 296, 580–583.

[26] Krishnamachary B, Glunde K, Wildes F, Mori N, Takagi T, Raman V, and Bhuwalla ZM (2009). Noninvasive Detection of Lentiviral-Mediated Choline Kinase Targeting in a Human Breast Cancer Xenograft. Cancer Res 69, 3464–3471.

[27] Gallarini M, Ferraris JD, and Burg MB (2008). GDPD5 is a glycerophosphocholine phosphodiesterase that osmotically regulates the osmoprotective organic osmolyte GPC. Proc Natl Acad Sci U S A 105, 11026–11031.

[28] Stewart JD, Marchan R, Lesjak MS, Lambert J, Hergenroeder R, Ellis JK, Lau C-H, Keun HC, Schmitz G, and Schiller J, et al (2012). Choline-releasing glycerophosphodiesterase EDI3 drives tumor cell migration and metastasis. Proc Natl Acad Sci U S A 109, 8155–8160.

[29] Cao MD, Döpflens M, Krishnamachary B, Vesuna F, Gadiya MM, Lannen PE, Bhuwalla ZM, Gribbestad IS, and Glunde K (2012). Glycerophosphodiester phosphodiesterase domain containing 5 (GDPD5) expression correlates with malignant choline phospholipid metabolite profiles in human breast cancer. NMR Biomed 25, 1033–1042.

[30] Cao MD, Cheng M, Rizwan A, Jiang L, Krishnamachary B, Bhuwalla ZM, Bathen TF, and Glunde K (2016). Targeting choline phospholipid metabolism: GDPD5 and GDPD6 silencing decrease breast cancer cell proliferation, migration, and invasion. NMR Biomed 29, 1098–1107.

[31] Chan KW, Jiang L, Cheng M, Wijnen JP, Liu G, Huang P, van Zijl PC, McMahon MT, and Glunde K (2016). CEST-MRI detects metabolite levels altered by breast cancer cell aggressiveness and chemotherapy response. NMR Biomed 29, 806–816.

[32] Robert J and Gianni L (1993). Pharmacokinetics and metabolism of anthracyclines. Cancer Surv 17, 219–252.

[33] Scott SA, Selvy PE, Buck JR, Cho HP, Criswell TL, Thomas AL, Armstrong MD, Arteaga CL, Lindsley CW, and Brown HA (2009). Design of isoform-selective phospholipase D inhibitors that modulate cancer cell invasiveness.Nat Chem Biol 5, 108–117.

[34] Bandypadhyay A, Wang L, Agrin J, Tang Y, Lin S, Yeh IT, De K, and Sun L-Z (2010). Doxorubicin in Combination with a Small TGFβ Inhibitor: A Potential Novel Therapy for Metastatic Breast Cancer in Mouse Models. PLoS One 5, e10365.

[35] Kim JH, H-w Kim, Jeon H, Suh P-G, and Ryu SH (2006). Phospholipase D1 Regulates Cell Migration in a Lipase Activity-independent Manner. J Biol Chem 281, 15747–15756.

[36] Morse DL, Raghunandan N, Sadarangani P, Murthi S, Job C, Day S, Howison C, and Gillies RJ (2007). Response of choline metabolites to docetaxel therapy is quantified in vivo by localized 31P MRS of human breast cancer xenografts and in vitro by high-resolution 31P NMR spectroscopy of cell extracts. Magn Reson Med 58, 270–280.

[37] Iorio E, Mezzanzanica D, Alberti P, Spadaro F, Ramoni C, D’Ascenzo S, Millimaggi D, Pavan A, Dolo V, and Canevari S, et al (2005). Alterations of Choline Phospholipid Metabolism in Ovarian Tumor Progression. Cancer Res 65, 9369–9376.

[38] Bolan PJ (2013). Magnetic Resonance Spectroscopy of the Breast: Current Status. Magn Reson Imaging Clin N Am 21, 625–639.

[39] Bolan PJ, Kim E, Herman BA, Newstead GM, Romen MA, Schnall MD, Pisano ED, Weatherall PT, Morris EA, and Lehman CD, et al (2016). Investigators AThr-MR spectroscopy of breast cancer for assessing early treatment response: Results from the ACRIN 6657 MRS trial. J Magn Reson Imaging http://dx.doi.org/10.1002/jmri.25560. [Epub ahead of print].

[40] Stelhouwer BL, van der Kemp WJ, Luijten PR, van den Bosch MA, Veldhuis WB, Wijnen JP, and Klomp DW (2014). (31)P magnetic resonance spectroscopy of the breast and the influence of the menstrual cycle. Breast Cancer Res Treat 144, 583–589.

[41] Cao MD, Lamichhane S, Lundgren S, Bojin A, Fjose H, Giskeodegard GF, and Bathen TF (2014). Metabolic characterization of triple negative breast cancer. BMC Cancer 14, 941.

[42] Fuss TL and Cheng LL (2016). Evaluation of Cancer Metabomics Using ex vivo High Resolution Magic Angle Spinning (HRMAS) Magnetic Resonance Spectroscopy (MRS). Metabolites 6 http://dx.doi.org/10.3390/metabo6010011.

[43] Wu CL, Jordman KW, Ratai EM, Sheng J, Adkins CB, Defeo EM, Jenkins BG, Ying L, McDougall WS, and Cheng LL (2010). Metabolomic imaging for human prostate cancer detection. Sci Transl Med 2, 16ra8.

[44] Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Dancey J, Arbuck S, Gwyther S, and Mooney M, et al (2009). New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer 45, 228–247.

[45] Beloueche-Babari M, Jackson LE, Al-Saffar NMS, Workman P, Leach MO, and Ronen SM (2005). Magnetic Resonance Spectroscopy Monitoring of Mitogen-Activated Protein Kinase Signaling Inhibition. Cancer Res 65, 3356–3363.

[46] Beloueche-Babari M, Peak JC, Jackson LE, Tier M-Y, Leach MO, and Eccles SA (2009). Changes in choline metabolism as potential biomarkers of phospholipase Cy1 inhibition in human prostate cancer cells. Cancer Res 69, 1305–1311.

[47] Ward CS, Eriksson P, Izquierdo-Garcia JL, Brandes AH, and Ronen SM (2013). HDAC Inhibition Induces Increased Choline Uptake and Elevated Phosphocho-line Levels in MCF7 Breast Cancer Cells. PLoS One 8, e62610.

[48] Mori N, Glunde K, Takagi T, Raman V, and Bhuwalla ZM (2007). Choline Kinase Down-regulation Increases the Effect of 5-Fluouracil in Breast Cancer Cells. Cancer Res 67, 11284–11290.

[49] de la Cueta A, Ramírez de Molina A, Álvarez-Ayerza N, Ramos MA, Gebrían A, Pulgar TGd and Lacal JC (2013). Combined 5-FU and ChoxK Inhibitors as a New Alternative Therapy of Colorectal Cancer: Evidence in Human Tumor-Derived Cell Lines and Mouse Xenografts. PLoS One 8, e64961.

[50] Arlauckas SP, Popov AV, and Delikanis EJ (2014). Direct inhibition of choline kinase by a near-infrared fluorescent carbocyanine. Mol Cancer Ther 13, 2149–2158.

[51] Hernandez-Alcoeza R, Saniger L, Campos J, Nunez MC, Khaless F, Gallo MA, and Arteaga CL, Lindsley CW, and Brown HA (2009). Design of isoform-selective phospholipase D inhibitors that modulate cancer cell invasiveness. Nat Chem Biol 5, 108–117.

[52] Bandypadhyay A, Wang L, Agrin J, Tang Y, Lin S, Yeh IT, De K, and Sun L-Z (2010). Doxorubicin in Combination with a Small TGFβ Inhibitor: A Potential Novel Therapy for Metastatic Breast Cancer in Mouse Models. PLoS One 5, e10365.

[53] Kim JH, H-w Kim, Jeon H, Suh P-G, and Ryu SH (2006). Phospholipase D1 Regulates Cell Migration in a Lipase Activity-independent Manner. J Biol Chem 281, 15747–15756.

[54] Morse DL, Raghunandan N, Sadarangani P, Murthi S, Job C, Day S, Howison C, and Gillies RJ (2007). Response of choline metabolites to docetaxel therapy is quantified in vivo by localized 31P MRS of human breast cancer xenografts and in vitro by high-resolution 31P NMR spectroscopy of cell extracts. Magn Reson Med 58, 270–280.

[55] Iorio E, Mezzanzanica D, Alberti P, Spadaro F, Ramoni C, D’Ascenzo S, Millimaggi D, Pavan A, Dolo V, and Canevari S, et al (2005). Alterations of Choline Phospholipid Metabolism in Ovarian Tumor Progression. Cancer Res 65, 9369–9376.

[56] Bolan PJ (2013). Magnetic Resonance Spectroscopy of the Breast: Current Status. Magn Reson Imaging Clin N Am 21, 625–639.