Published in final edited form as:

Mol Cancer Ther. 2008 August ; 7(8): 2556–2565. doi:10.1158/1535-7163.MCT-08-0015.

Fatty acid synthase inhibition results in a magnetic resonance-detectable drop in phosphocholine

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Abstract

Expression of fatty acid synthase (FASN), the key enzyme in *de novo* synthesis of long-chain fatty acids (FA), is normally low but increases in cancer. Consequently, FASN is a novel target for cancer therapy. However, because FASN inhibitors can lead to tumor stasis rather than shrinkage, non-invasive methods for assessing FASN inhibition are needed. To this end, we combined ¹H, ³¹P and ¹³C magnetic resonance spectroscopy (MRS) (i) to monitor the metabolic consequences of FASN inhibition and (ii) to identify MRS-detectable metabolic biomarkers of response. Treatment of PC-3 cells with the FASN inhibitor Orlistat for up to 48 h resulted in inhibition of FASN activity by 70%, correlating with 74% inhibition of FA synthesis. Furthermore, we have determined that FASN inhibition results not only in lower phosphatidylcholine levels, but also in a 59% drop in the phospholipid precursor phosphocholine (PCho). This drop resulted from inhibition in PCho synthesis as a result of a reduction in the cellular activity of its synthetic enzyme choline kinase. The drop in PCho levels following FASN inhibition was confirmed in SKOV-3 ovarian cancer cells treated with Orlistat and in MCF-7 breast cancer cells treated with Orlistat as well as cerulenin. Combining data from all treated cells, the drop in PCho significantly correlated with the drop in *de novo* synthesized FA levels, identifying PCho as a potential non-invasive MRS-detectable biomarker of FASN inhibition *in vivo*.

Keywords

fatty acid synthase; magnetic resonance spectroscopy; choline metabolism; Orlistat; choline kinase

Introduction

Fatty acid synthase (FASN) is the key enzyme in the *de novo* synthetic pathway of long-chain fatty acids (FA) (1). In most normal cells, FASN expression is low and FA are obtained from the diet (2). However, FASN expression is significantly increased in a wide variety of human cancers including prostate, breast, colon and ovarian cancer (3–7). Furthermore, this over-expression is associated with poor prognosis, particularly in the case of breast and prostate cancer (4,8).

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In light of these observations, FASN has been proposed as a novel target for cancer therapy (9–11). Indeed, recent studies show that inhibition of FASN by pharmacological (e.g. Orlistat, cerulenin, C75, EGCG) or siRNA treatments result in cell cycle arrest and apoptosis of transformed cells *in vitro*, while *in vivo* studies show that treatment with FASN inhibitors results in inhibition of tumor growth (12–17). Importantly, normal epithelial cells are not affected by FASN inhibition (9,12). Consequently, the use of FASN inhibitors, as well as inhibitors of other enzymes involved in FA synthesis (18–20), present a promising therapeutic approach.

However, because response to FASN inhibitors can result in tumor stasis rather than tumor shrinkage, conventional imaging methods may not be adequate to rapidly assess therapeutic response. Consequently, additional non-invasive methods for monitoring inhibition of FA synthesis are needed. Furthermore, whereas the direct consequences of FASN inhibition on FA synthesis, and the subsequent modulation of membrane phosphatidylcholine (PtdCho) levels have been investigated in detail (12,13,21), additional studies are required to assess further effects of FASN inhibitors on other aspects of cellular metabolism.

Magnetic resonance spectroscopy (MRS) is a non-invasive, non-destructive method that can provide longitudinal information regarding tumor metabolism as well as its modulation following treatment. It has previously been used to monitor choline phospholipid metabolism, glucose metabolism and cellular energy levels as well as response to chemotherapeutic agents and therapies targeted to specific oncogenic pathways (22–28). As such, MRS can therefore provide a method both for investigating the overall metabolic consequences of FASN inhibition and for non-invasively assessing the molecular action of FASN inhibitors *in vivo*.

The goal of the work described here was therefore twofold. First, using MRS, to simultaneously determine the effect of FASN inhibitors on FA synthesis and on other metabolic pathways. Second, using the MRS findings, to identify a MRS-based, non-invasive, metabolic biomarker of FASN inhibition that, in the long term, could be used to monitor FASN inhibition *in vivo*. To this end, we have used ^1H , ^{31}P and ^{13}C MRS to investigate the metabolic consequences of FASN inhibition. Our observations are in line with previous reports showing that in addition to inhibiting *de novo* synthesis of FA, FASN inhibition also leads to a drop in membrane PtdCho levels. However, we also show, to our knowledge for the first time, that FASN inhibition also results in a drop in *de novo* synthesis of the PtdCho precursor phosphocholine (PCho), and a drop in cellular PCho levels. Importantly, the drop in PCho was correlated with the drop in *de novo* synthesized FA levels, identifying PCho as a potential MRS-based metabolic biomarker of FASN inhibition.

Materials and Methods

Cell culture and FASN inhibition

PC-3 human prostate, MCF-7 human breast and SKOV-3 human ovarian cancer cells were routinely cultured in DMEM/F12 (Gibco, NY, USA) supplemented with 10% heat-inactivated FBS (Hyclone, UT, USA) and 100 U/mL penicillin 100 µg/mL streptomycin, 0.25 µg/mL amphotericin (Gibco, NY, USA) and 2 mM L-glutamine (Cellgro, VA, USA) at 37 °C in 5% CO₂. For all FASN inhibition studies, FBS was lowered to 5% (in order to limit the amount of available extra-cellular FA) and glucose in the medium was reduced by half to 8.76 mM (financial reasons). To inhibit FASN, PC-3 cells were incubated for 24 h and 48 h with 30 µM Orlistat (treated) or with carrier dimethylsulfoxide (DMSO) at 0.5% v/v (control cells). MCF-7 and SKOV-3 cells were incubated for 48 h with 30 µM Orlistat (treated) or with DMSO at 0.5% v/v (control cells). MCF-7 cells were also treated with 30 µM cerulenin (treated) or with DMSO at 0.5% v/v (control cells).

FASN activity assay

FASN activity was determined as previously described (29). Briefly, $\sim 5 \times 10^6$ cells were trypsinized, washed in PBS and frozen at -80°C . Cells were resuspended in 1 mL of lysis buffer containing 1 mM EDTA, 150 mM NaCl, 100 $\mu\text{g/mL}$ PMSF and 50 mM Tris-HCl at pH 7.3, subjected to vortex for 30 s at 0°C and disrupted at 0°C by ultrasonication for 10 periods of 1 s. The lysates were centrifuged ($16,000 \times g$, 15 min) and the supernatant stored at -80°C and assayed within 1 week. A sample was taken to measure protein content (Bio-Rad DC protein assay, Bio-Rad Laboratories, CA, USA). Lyophilized supernatant ($\sim 5 \mu\text{g}$) was added to a mixture of 200 mM potassium phosphate buffer (pH 6.6), 1 mM dithiothreitol, 1 mM EDTA, 0.24 mM NADPH and 30 μM acetyl-CoA in 0.2 mL reaction volume. After monitoring at 340 nm (Beckman Coulter DU800 UV/Visible spectrophotometer, CA, USA) at room temperature for 3 min to measure background NADPH oxidation, 50 μM of malonyl-CoA was added and the reaction mixture assayed for 10 min. Data was analyzed using Beckman Coulter DU800 System and Applications Software.

Cell cycle analysis

Flow cytometry was used to determine the effect of FASN inhibition on cell cycle as follows. $\sim 2 \times 10^6$ PC-3 and MCF-7 trypsinized cells were fixed in 95% ice-cold ethanol and then stained with a PBS solution containing 40 $\mu\text{g/mL}$ propidium iodide (PI) (Sigma-Aldrich, MO, USA) and 100 $\mu\text{g/mL}$ RNase A (Sigma-Aldrich, MO, USA). Cells were analyzed using a BD FACSCalibur (Becton Dickinson, CA, USA) instrument using a 488 nm excitation wavelength. Cells of uniform width were gated and PI intensities were plotted and analyzed using ModFit LT (version 3.1) software (Topsham, ME, USA). In the case of PC-3 cells, the integration values of the two major G1 populations (diploid (2n) and aneuploid (2.8n)) within the culture were summed to derive the final cell cycle distribution results. The values for their corresponding G2 (4n and 5.6n) and S phase populations were also combined.

Apoptosis assay

PC-3 and MCF-7 cells (1×10^5) were plated in 4 mm² wells, incubated overnight, and treated with 30 μM Orlistat for 3, 24, and 48 hours. At each time-point, the media was removed and transferred to a 1.5 mL tube to preserve any floating cells. The cell monolayers were then washed once with PBS, trypsinized, resuspended in 0.5 mL PBS and transferred to the corresponding media tubes. The cell suspensions were then centrifuged at $1000 \times g$ for 5 minutes, washed once with PBS and lysed using 100 μL of lysis buffer (50 mM HEPES, pH 7.4, 0.1% CHAPS, 5 mM DTT, 0.1 mM EDTA). The lysates were centrifuged at $10,000 \times g$ for 10 minutes, and stored at -20°C . Caspase-3 activity was measured by incubating 10 μL of each lysate with 30 μM Ac-DEVD-AMC substrate (Biomol, Plymouth Meeting, PA) in assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol) in a 100 μL total volume for 1 hour at 37°C . Cleavage of the substrate by Caspase-3 was measured using a Safire plate reader (Tecan Group, Switzerland) at excitation and emission wavelengths of 360nm and 460nm, respectively.

Cell extraction, MRS acquisition and analysis

For MRS studies, glucose in the medium (8.76 mM) was replaced by equal concentrations of 1-¹³C-glucose and unlabeled glucose (to monitor FA synthesis and glycolysis) and choline in the medium was replaced with 1,2-¹³C-choline at its normal concentration of 64.1 μM (to monitor choline metabolism). PC-3, MCF-7 or SKOV-3 cells (logarithmic growth) were cultured with Orlistat (treated) or DMSO (control) for 24 h and/or 48 h, replenishing medium every 24 h. Spent medium was saved. To monitor cell metabolism, $\sim 5 \times 10^7$ cells were extracted using the dual-phase extraction method (25,30). Briefly, cells were rinsed with ice-cold saline, fixed in 10 mL of ice-cold methanol and scraped from the culture flask surface.

Alternatively, cells were trypsinized, centrifuged and the cell pellet combined with 10 mL of ice-cold methanol (results from both methods were indistinguishable). Following vortexing, 10 mL of ice-cold chloroform was added followed by 10 mL of ice-cold de-ionized water. After phase separation and solvent removal, cellular proteins were collected and stored at -80°C until measurement (see below). To acquire ^1H and ^{13}C spectra, the aqueous fraction was reconstituted in 500 μL of deuterium oxide (D_2O , aqueous phase) and the lipid fraction was reconstituted in deuterated chloroform (CDCl_3 , lipid phase). To acquire aqueous ^{31}P MR measurements, 25 μL of EDTA in D_2O were further added to a final concentration of 5 mM. To acquire lipid ^{31}P MR measurements, CDCl_3 was evaporated and the precipitate re-suspended in 500 μL of a 2:1 mixture of CDCl_3 and 60 mM methanolic EDTA adjusted to pH 7.3 with CsOH .

MR spectra were acquired on an Avance DRX500 Bruker spectrometer (Bruker Biospin, Germany). ^1H spectra: 20 ppm spectral width, 30° flip angle and 5 second repetition time with water suppression. ^{13}C spectra: 240 ppm spectral width, 30° flip angle and 3.5 second repetition time with broad-band proton decoupling. ^{31}P spectra: 60 ppm spectral width, (aqueous ^{31}P) or 35 ppm (lipid ^{31}P), 30° flip angle, 4.5 second repetition time and broad-band proton decoupling. Data were analyzed using Bruker Topspin 2.0.a. software. Changes in metabolite levels were determined relative to matched controls. Absolute metabolite quantification was obtained by determining the appropriate peak area, normalizing to an external reference (tetramethylsilane (TMS), ^1H MRS; CDCl_3 , ^{13}C MRS; methylenediphosphonic acid (MDPA), ^{31}P MRS), correcting for saturation and nuclear Overhauser effects (NOE) and normalizing to cellular protein. In quantifying the product of *de novo* FA synthesis (^{13}C MR spectra) only the methylene carbons at 29–30 ppm were used and it was further assumed that FA observed represented only palmitate. From ^1H MR spectra of lipid extracts only methylene protons at 1.2–1.3 ppm were used to quantify the total pool of FA and all FA were assumed to be palmitate.

To quantify the cellular proteins that precipitated during metabolite extraction, the pellet was re-suspended in 1 mL of 1 M NaOH by incubating for 1 h at 50°C . A sample was then taken for measurement of protein content (Bio-Rad assay, Bio-Rad Laboratories).

Choline kinase assay

Choline kinase (ChoK) activity was determined as described (31). Following incubation with DMSO or Orlistat for 48 h, $\sim 15 \times 10^6$ PC-3 cells were trypsinized, washed in ice-cold PBS and re-suspended in 400 μL of Tris-HCl (pH 8.0) containing 10 mM dithiothreitol and 1 mM EDTA in D_2O . Cells were homogenized for 30 s at 0°C and were disrupted at 0°C by ultrasonication for 10 periods of 1 second. Cell lysates were centrifuged ($16,000 \times g$, 30 min) and the particle-free supernatant transferred to a NMR tube. Initial PCho levels were measured by ^1H MRS as described above. ChoK activity was assayed by monitoring temporal accumulation of PCho by MRS following addition of choline chloride, ATP and Mg^{2+} in Tris-HCl buffer (final concentrations: 5 mM choline chloride, 10 mM ATP, 10 mM MgCl_2). ChoK activity was determined from a straight line fit to plots of PCho as nmol/mg protein versus time following addition of all substrates.

The effect of Orlistat on ChoK activity was also monitored after only 1 h of incubation. Cells were incubated for 47 h with DMSO alone, then Orlistat added during the last hour of incubation, creating a treated group. Cells were then lysed and ChoK activity assayed as before. To detect any direct interaction between Orlistat and ChoK, cells were incubated for 47 h with DMSO alone. Following extraction, half the cell lysates were exposed to Orlistat for 1 h at room temperature, creating a treated group, and ChoK activity assayed as before.

Quantitative PCR (q PCR) Analysis—PC-3 cells (2.5×10^5) were seeded in 10 cm^2 dishes for 24 hours before treating with 30 μM Orlistat. RNA from treated and control cells was

extracted cells using TRIZOL (Invitrogen, Carlsbad, CA) and standardized to a concentration of 50 ng/mL. cDNA was produced using a Transcriptor First Strand synthesis kit (Roche, Mannheim, Germany) according to manufacturer's instruction. The expression of phosphocholine cytidyltransferase (CCT) and ChoK following treatment was measured by quantitative PCR (qPCR) using an ABI 7500 instrument (Applied Biosystems, Foster City, CA). PCR primers were designed based on GenBank sequences NM_005017 (CCT; Forward 5'-TGTTTCAGCCAAGGTCAATGCAAGG-3' and Reverse 5'-TTCTCGTTCATCACCGTGAAGCCT-3') and NM_001277 (choline kinase; Forward 5'-TATCTTGTGTGCTGGAAGGCCGAGA-3' and Reverse 5'-TGGGCGTAGTCCATGTACCCAAAT-3'). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous standard and amplified using primers 5'-CCATGGAGAAGGCTGGGG-3' (Forward) and 5'-CAAAGTTGTCATGGATGACC-3' (Reverse). Expression levels of CCT and choline kinase were first normalized to endogenous GAPDH levels (ΔCt) and subsequently to the untreated control of each group ($\Delta\Delta\text{Ct}$). The results were expressed as percentages of relevant control using the equation $2^{-\Delta\Delta\text{Ct}}$.

Western blot analysis

PC-3 and MCF-7 cells were treated with DMSO or Orlistat (30 μM) for 3 hrs, 24 hrs and 48 hrs, then lysed using cell lysis buffer containing 1% NP40, 1% SDS and 1 $\mu\text{L}/\text{mL}$ protease inhibitor cocktail set III (Calbiochem, La Jolla, CA). Lysates were centrifuged at 12,000 rpm for 10 minutes at 4°C, the protein supernatant was collected and total protein concentrations were determined using Bio-Rad DC protein assay reagents (Bio-Rad, Hercules, CA). Proteins were separated by SDS-PAGE using 10% gels and transferred electrophoretically to 0.45 μm nitrocellulose membranes. Membranes were blocked in blocking buffer containing 5% nonfat dry milk in TBS (pH 7.6) and 0.1% Tween 20 and incubated overnight at 4°C with primary antibodies as follows: Akt (1:1,000; Cell Signaling Technology, Danvers, MA), Phospho-Akt (P-Akt, 1:1,000; Cell Signaling Technology) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5,000; Stressgen). This was followed by 1-hour incubation with horse radish peroxidase-conjugated secondary anti-rabbit (Cell Signaling Technology) and anti-mouse (Cell Signaling Technology) antibodies at dilutions of 1:1,000 and 1:2,000 respectively. Membranes were washed with enhanced chemiluminescence reagents (LumiGLO & Peroxide, Cell Signaling Technology) for 1 minute and exposed to Hyperfilm (Amersham Biosciences, Piscataway, NJ), which was developed on a Konica SRX-101 automatic developer (Konica, Tokyo, Japan). The intensity of individual bands of interest was quantified using Image J software (National Institutes of Health, Bethesda, MD) and normalized to the intensity of glyceraldehyde-3-phosphate dehydrogenase (loading control) and Akt and P-Akt protein expressions compared between DMSO and Orlistat treated groups.

Statistical analysis

Unless otherwise stated, experiments were repeated 3–5 times. Results are expressed as mean $\pm$ SD. Two-tailed unpaired Student's *t* tests were performed to assess the statistical significance of results. *P* value < 0.05 was considered significant.

Results

Orlistat treatment leads to inhibition of FA synthesis, reduced FA levels, inhibition in cell proliferation and lower NTP levels

First we investigated in detail the effect of the FASN inhibitor Orlistat on PC-3 prostate cancer cells (known to over-express FASN (7)). Cells were treated for 24 h and 48 h with 30 μM (saturating dose (12)) of Orlistat. As expected, this resulted in inhibition of FASN activity by $51 \pm 10\%$ ($P < 0.05$) and $70 \pm 21\%$ ($P < 0.05$) at 24 h and 48 h respectively when compared to controls (Figure 1A). Figure 1B illustrates ^{13}C MRS spectra obtained from the lipid fraction

of control and treated PC-3 cells grown in the presence of medium containing ^{13}C -labeled choline and glucose. From this data it was possible to monitor FA synthesized *de novo* from the ^{13}C labeled glucose present in the growth medium. As expected, FA synthesis was inhibited following Orlistat treatment. Since cellular protein content ($1.9 \pm 0.2 \times 10^{-7}$ mg/cell) was unaffected by Orlistat treatment, metabolite levels, determined by MRS, were normalized to protein content. Control cells synthesized 30 ± 12 nmol/mg protein of *de novo* ^{13}C labeled FA during 24 h and 56 ± 18 nmol/mg protein during 48 h. In cells treated with Orlistat, *de novo* ^{13}C labeled FA synthesis decreased significantly by $45 \pm 21\%$ to 15 ± 2 nmol/mg protein ($P < 0.05$) at 24 h and by $74 \pm 12\%$ to 13 ± 4 nmol/mg protein ($P < 0.02$) at 48 h. It is noteworthy that the measured decrease in *de novo* FA synthesis correlated with the measured decrease in FASN activity following 24 h and 48 h of Orlistat treatment ($R^2 = 0.99$, $P < 0.05$). Figure 1C illustrates the ^1H MRS spectra of the same cellular extracts monitoring the total FA pool. Assuming that on average all FA are 16 carbons in length, the cellular FA pool was estimated as 523 ± 147 nmol/mg protein in control PC-3 cells. Following Orlistat treatment, and consistent with the inhibition of *de novo* FA synthesis, total cellular FA levels dropped slightly to $91 \pm 31\%$ following 24 h and significantly to $70 \pm 17\%$ ($P < 0.04$) following 48 h of FASN inhibition.

Orlistat treatment also led to cell cycle arrest in PC-3 (Figure 2A). Cell fractions in the G1 phase increased in association with length of treatment. A matched concomitant decrease in the S and G2-M phase fractions was also observed in both cell lines. Cell cycle arrest was associated with inhibition of cell proliferation. Thus, the number of Orlistat-treated PC-3 cells remained essentially unchanged at $111 \pm 17\%$ at 24 h and $83 \pm 11\%$ at 48 h compared to cell numbers prior to treatment. In contrast, untreated control cell numbers increased to $149 \pm 38\%$ ($P < 0.05$ relative to treated cells) and $206 \pm 62\%$ ($P < 0.005$ relative to treated cells) at 24 h and 48 h respectively. Apoptosis was not evident during the course of Orlistat treatment. Specifically, no significant increase in apoptotic cells was observed in treated cells, where the maximum percentage of the apoptotic cell fraction (sub-G1) did not exceed $0.9 \pm 0.3\%$ in treated PC-3 cells. Furthermore, an increase in Caspase-3 activity was not detected at 3, 24, and 48 hours post-treatment, in contrast to the significant increase detected in a positive control, staurosporine-treated Jurkat cells (data not shown).

Levels of the high-energy metabolites phosphocreatine (PCr) and nucleotide triphosphates (NTP - predominantly consisting of ATP) were determined from ^{31}P MR spectra of the aqueous fraction of control PC-3 extracts at values measuring 16 ± 7 nmol/mg protein and 57 ± 22 nmol/mg protein, respectively. PCr levels decreased significantly following 24 h of Orlistat treatment to below detection level, where they remained through 48 h of FASN inhibition. NTP levels also decreased following treatment, with their levels falling significantly by $34 \pm 23\%$ ($P < 0.03$) and $59 \pm 10\%$ ($P < 0.002$) at 24 h and 48 h, respectively (Figure 3A).

Orlistat treatment leads to a drop in phosphocholine levels

Levels of the membrane lipid PtdCho were determined from the ^{31}P MR spectra of the lipid fraction of cell extracts (Figure 4A). Changes in the levels of this phospholipid were below detection level during 24 h of Orlistat treatment, with values at $110 \pm 44\%$ ($P = 0.74$) relative to control. However at 48 h or treatment PtdCho dropped by $31 \pm 4\%$ ($P < 0.0005$) from 115 ± 31 to 80 ± 24 nmol/mg protein following 48 h of treatment, consistent with previous reports (12, 13, 21). ^{13}C MRS studies (Figure 4B) indicated that this was due to a reduction in *de novo* phospholipid synthesis. Control cells synthesized 21 ± 6 nmol/mg protein and 61 ± 12 nmol/mg protein PtdCho during 24 h and 48 h, respectively. Following Orlistat treatment for 24 h, PtdCho synthesis decreased by $54 \pm 24\%$ to 10 ± 4 nmol/mg protein ($P < 0.03$) and by $60 \pm 9\%$ to 25 ± 9 nmol/mg protein ($P < 0.004$) following 48 h of treatment.

In addition, and somewhat unexpectedly, ^{31}P MRS spectra of the aqueous fraction of cell extracts indicated that levels of total PCho, the precursor of PtdCho, were also affected by

Orlistat treatment (Figure 3A). PCho dropped significantly by $41\pm24\%$ from an average 182 ± 20 nmol/mg protein to 107 ± 44 nmol/mg protein following 24 h of Orlistat treatment ($P<0.02$) and by $60\pm21\%$ from 199 ± 63 nmol/mg to 70 ± 26 nmol/mg protein ($P<0.02$) following 48 h of treatment. The total choline-containing metabolite signal (tCho) detected by ^1H MRS (data not shown) also dropped by $33\pm27\%$ ($P=0.05$) and by $47\pm32\%$ ($P=0.06$) at 24 and 48 h respectively, further confirming the effect of FASN inhibition on PCho levels. ^{13}C MRS studies were used to study PCho synthesis simultaneously with FA synthesis and figure 3B illustrates the ^{13}C spectra of control and Orlistat-treated cells. These studies indicated that the drop in PCho levels, was due to its reduced synthesis. Control cells synthesized 83 ± 13 nmol/mg protein of *de novo* PCho during 24 h and 131 ± 27 nmol/mg protein during 48 h. Synthesis decreased significantly upon treatment with Orlistat by $39\pm12\%$ to 47 ± 11 nmol/mg protein and by $59\pm23\%$ to 37 ± 15 nmol/mg protein after 24 h and 48 h respectively. Importantly, total as well as *de novo* PCho levels correlated with FASN activity ($R^2 = 0.99$, $P<0.05$).

The drop in phosphocholine is associated with a drop in cellular choline kinase activity

The data above indicate that following 48 h of Orlistat treatment the drop in total PCho levels (129 ± 89 nmol/mg protein) was comparable to the drop in PCho synthesized *de novo* (94 ± 42 nmol/mg protein). This indicated that the drop in PCho levels was due to reduced PCho synthesis, rather than increased utilization. To confirm this hypothesis, ChoK activity was assayed in control and treated PC-3 cells using MRS, as previously described (31). The rate of PCho synthesis, a measure of cellular ChoK activity, was 34 ± 6 nmol/mg protein·h in control cells. Following FASN inhibition with Orlistat for 48 h this rate dropped significantly to 21 ± 7 nmol/mg protein·h ($P<0.05$) (Figure 5A).

We questioned how quickly Orlistat affects choline metabolism, and to this end also monitored ChoK activity following 1 hour of Orlistat treatment. At that time point, FASN inhibition resulted in a drop in ChoK activity by $49\pm10\%$ ($P<0.05$) relative to controls. This inhibition was comparable to that observed following 48 h of treatment and indicated that the effect of FASN inhibition on choline metabolism occurs rapidly and is sustained during treatment.

To rule out any direct inhibitory interaction between Orlistat and ChoK, Orlistat was also added directly to the extracted cell lysates. Incubation of cell lysates with $30\text{ }\mu\text{M}$ Orlistat for 1 h did not alter the activity of ChoK, which remained at $110\pm36\%$ ($P=0.67$) of control levels.

To determine the reasons behind the drop in cellular ChoK activity, we monitored enzyme expression. qPCR analysis demonstrated that ChoK expression was unchanged at 24 h with gene expression at $106\pm4\%$ relative to control. However, 48 h of Orlistat treatment resulted in a drop in ChoK expression to $60\pm28\%$ ($P<0.05$) relative to controls. The expression of CCT was not significantly altered during the same time with qPCR data showing CCT expression at $95\pm9\%$ and $99\pm10\%$ at 24h and 48h respectively. Thus, changes in ChoK expression can explain the drop in PCho levels following 48 h of treatment, but not the drop in ChoK activity and PCho levels observed at the earlier time points following FASN inhibition.

PI3K signaling has been linked to FASN expression in clinical samples (11,32,33) and, recently, a bi-directional link between FASN and PI3K signaling has been shown, with PI3K controlling FASN expression and FASN inhibition resulting in a reduction in PI3K signaling downstream of her2/neu (11,34,35). We have previously shown that inhibition of the PI3K pathway results in a drop in PCho levels (36), and ChoK activity can be controlled by PI3K signaling (37). We therefore questioned whether PI3K signaling provided the mechanistic link between FA and choline metabolism and could explain the drop in ChoK activity and PCho levels following FASN inhibition, particularly at the earlier time points. To answer this question we monitored the effect of Orlistat on P-Akt levels in PC-3. A slight drop in P-Akt levels was observed following 3 hours of Orlistat treatment in PC-3 cells (Fig. 5B) indicating

some inhibition of PI3K signaling. However 24 or 48 hours of exposure to Orlistat did not result in a significant reduction in P-Akt levels.

The drop in phosphocholine is a general observation following FASN inhibition

To confirm the generality of our observations we extended our studies of Orlistat to another two cell lines - MCF-7 human breast cancer and SKOV-3 human ovarian cancer cells. We further extended our studies to a second FASN inhibitor - cerulenin. Our findings are summarized in Table 1. MCF-7 cells were treated with 30 μ M Orlistat for 48 h. This led to a drop in cell numbers to $57\pm14\%$ compared to controls, associated with cell cycle arrest and no significant apoptosis, as in the case of PC-3 cells (Figure 2B). As expected, FASN inhibition resulted in a drop in *de novo* FA synthesis by $46\pm9\%$ in MCF-7 cells. Importantly however, the inhibition of FASN activity was associated, as in the case of PC-3 cells, with a drop in *de novo* synthesized PCho levels by $63\pm12\%$ resulting in a drop in total PCho levels by $38\pm6\%$. Levels of tCho also dropped by $49\pm15\%$ in MCF-7 cells. The effect of Orlistat treatment on cellular energetics was less substantial than in PC-3 cells, with NTP levels falling by $28\pm32\%$. Western blot analysis of P-Akt levels in MCF-7 cells yielded similar results to PC-3 cells, with no significant inhibition of PI3K signaling observed following 48 hours of Orlistat treatment.

Treatment of SKOV-3 cells with Orlistat also led to inhibition of PCho synthesis and a reduction in PCho levels (Table 1). Specifically, FASN inhibition resulted in a drop in SKOV-3 cell number to $79\pm7\%$ relative to control associated with a drop in *de novo* FA synthesis by $37\pm7\%$, a drop in *de novo* synthesized PCho levels by $31\pm21\%$, and a drop in total PCho levels by $56\pm14\%$.

MCF-7 cells were also treated with 30 μ M cerulenin for 48 h (Table 1). The effect on cell proliferation was negligible, with cell numbers remaining at $99\pm17\%$ of control. Nonetheless, treatment with cerulenin was associated with a reduction in *de novo* FA synthesis by $40\pm6\%$. Furthermore, as in the case of Orlistat, the inhibition of FASN activity was associated with a drop in *de novo* synthesized PCho levels by $21\pm12\%$ and, importantly, total PCho levels dropped by $32\pm14\%$ in cerulenin-treated MCF-7 cells while tCho dropped by $48\pm4\%$. Cerulenin treatment had no noticeable effects on cellular energetics, with NTP levels remaining at $97\pm32\%$.

The drop in phosphocholine levels correlates with the drop in fatty acid synthesis following FASN inhibition

Finally, we wanted to confirm that PCho levels were correlated with FASN activity. As mentioned above, FASN activity tightly correlates with *de novo* synthesis of FA. We therefore used changes in *de novo* synthesis of FA as an indicator of FASN activity in the exact same experiment in which PCho levels were measured. Our findings are summarized in Figure 3C in which we combined the data obtained from the different experiments summarized in Table 1. We find that the drop in PCho levels significantly correlates with the drop in *de novo* FA synthesis ($R^2=0.73$; $P<0.03$), validating PCho as an indicator of FASN inhibition.

Discussion

FASN is over-expressed in several cancers, and inhibition of FASN leads to cell cycle arrest, apoptosis and inhibition of tumor growth, presenting a novel therapeutic approach for cancer treatment (9–17). Here, we monitored the metabolic consequences of FASN inhibition with the goal of determining the effects of this inhibition on cellular metabolism and identifying MR-based metabolic biomarkers of the molecular action of FASN inhibitors. We used ^1H , ^{31}P and ^{13}C MRS, combined with ^{13}C -labeled glucose and choline, to assess FA synthesis, choline metabolism and cellular energetics. First, using Orlistat, we determined in

detail the MRS-detectable consequence of FASN inhibition in PC-3 cells. We then confirmed the generality of our findings by investigating the effect of Orlistat on two other cell lines and the effect of an additional FASN inhibitor. As expected, FASN inhibition in all three cell lines resulted in inhibition of *de novo* FA synthesis and a drop in total FA levels which was generally associated with inhibition in cell proliferation. These findings are consistent with previous reports (12,13,15–17,38–41). Our MRS data also showed that FASN inhibition affects cellular energetics, with levels of PCr (when detectable) and NTP generally decreasing with FASN inhibition. However, this effect was not always large enough to be deemed a reliable indicator of FASN activity.

Since this study used MRS, where the metabolic fate of different precursors can be monitored simultaneously, it was possible to monitor choline metabolism at the same time as FA synthesis. Choline is the precursor of the main membrane phospholipid PtdCho in the Kennedy (CDP-choline) pathway. A drop in PtdCho levels has been previously reported (12,13,21) and others have shown a link between FA synthesis and the Kennedy pathway through transcriptional and posttranscriptional control of the rate limiting enzyme CCT (42). Our studies also showed a drop in PtdCho levels. However, in this study we observed, to our knowledge for the first time, that inhibition of FA synthesis also results in inhibition of the first step in this pathway, namely synthesis of PCho by ChoK. As a result, PCho levels were lower following treatment and, importantly, PCho levels, correlate with FA synthesis levels. Thus, we propose that a reduction in levels of the MRS-detectable metabolite PCho can be used as a non-invasive diagnostic indicator of FASN inhibition *in vivo*.

Regarding the mechanism for the observed drop in PCho, we hypothesized that FASN inhibition and inhibition of FA synthesis lead to upstream inhibition of the first enzyme in the Kennedy pathway, namely ChoK. To test this hypothesis, we monitored the cellular activity of ChoK and our data confirms that cellular ChoK activity is indeed significantly reduced following FASN inhibition. This modulation of ChoK activity results, at least in part, from the reduction in ChoK expression observed following 48 hr of Orlistat treatment. However, the fact that the activity of ChoK was reduced prior to this time point (within 1 h of treatment) indicates that other factors are also involved in modulating ChoK activity following FASN inhibition. We thus considered the possibility that cellular ChoK activity was also modulated by inhibition of PI3K signaling, reported to occur downstream of FASN inhibition (11,35, 37). In line with previous reports, our data indicated that PI3K signaling was indeed reduced following FASN inhibition. However, this effect was only observed following FASN inhibition for a relatively short time (3 hours) and was not sustained for 24 hr or 48 hr. Our data therefore indicate that most likely multiple translational and posttranslational factors are involved in modulating ChoK activity following FASN inhibition. It is possible that modulation of ChoK activity could be mediated by PI3K shortly after FASN inhibition, while the effects of longer-term treatment could be mediated by altered ChoK expression.

In conclusion, this study highlights the value of MRS for simultaneously monitoring the effect of one inhibitor on several metabolic pathways. Furthermore, our data in three different cell lines and using two FASN inhibitors indicates that a drop in PCho levels is correlated with inhibition of FA synthesis following FASN inhibition. MRS is a non-invasive method that can be used in patients *in vivo* to monitor PCho either by monitoring the phosphomonester peak in the ³¹P spectrum or, more commonly, by monitoring the total choline signal in the ¹H spectrum. Consequently, we propose that this previously unreported metabolic change could serve as a non-invasive *in vivo* MRS metabolic pharmacodynamic biomarkers of FASN inhibition.

Acknowledgements

We would like to acknowledge the NCI Cancer Center Support Grant CA016672 for the support of Core NMR facility. We thank Wendy Schober (MD Anderson Cancer Center core facility) for help in flow cytometry analysis.

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Figure 1.

Inhibition of FASN causes a drop in FA synthesis and eventually a drop in total FA levels.

A, % (relative to control) FASN activity (*black columns*), *de novo* FA synthesis (*white columns*) and total FA levels (*striped columns*) of PC-3 prostate cancer cells following treatment with Orlistat for the indicated times. Error bars, SD. *, $P < 0.05$, statistically significant difference from its control; two-tailed unpaired t test. *B*, representative ^{13}C MR spectra of lipid-phase extracts of PC-3 cells showing the effect of 48 h Orlistat treatment on *de novo* FA synthesis. All peaks shown represent FA but only the peak labeled “FA” was used for quantitative analysis. *C*, representative ^1H MR spectra of the lipid-phase extracts of PC-3 cells showing effect of 48 h Orlistat treatment on cellular FA levels.

Figure 2.

FASN inhibition results in cell cycle arrest without the induction of apoptosis in PC-3 (A) and MCF-7 (B) cells. Left: representative histograms showing cell cycle distribution of cells treated with Orlistat (30 μ M) for 24 and 48 h. The PC-3 cell line exhibits two major G1 populations, diploid (2n) and aneuploid (2.8n), and two corresponding G2-M peaks. Right: Cell cycle phase distributions shown in histograms were quantified using ModFit LT and the results are illustrated in the bar graphs. Each value was determined from the average of 3 analyses. Error bars, SD.

Figure 3.

Inhibition of FASN causes a drop in PCho synthesis leading to a decrease in total PCho levels which is correlated with a drop in FA synthesis. A, representative ^{31}P MR spectra of the aqueous extracts of PC-3 cells showing the effect of 48 h of Orlistat treatment on cellular PCho levels and cellular energetics. *Inset*: % (relative to control) cellular PCho levels of PC-3 cells following treatment with Orlistat for the times indicated. B, representative ^{13}C MR spectra of the aqueous extracts of PC-3 cells following Orlistat treatment for 48 h on *de novo* PCho synthesis. *Inset*: % (relative to control) *de novo* PCho synthesis in PC-3 cells following Orlistat treatment for the indicated times. Error bars, SD. *, $P < 0.05$. C. PCho levels as a function of de novo FA synthesis based on experiments summarized in Table 1 and indicating a correlation between FASN activity as indicated by *de novo* FA synthesis and PCho levels ($R^2 = 0.73$; $P < 0.03$).

Figure 4.

Inhibition of FASN causes a drop in the synthesis and total levels of membrane phospholipids. A, representative ³¹P MR spectra of PC-3 lipid extracts following 48 h of treatment with Orlistat; *a*, cardiolipin; *b*, plasmalogen-PtdEtn; *c*, PtdEtn; *d*, PtdS; *e*, sphingomyelin; *f*, phosphatidylinositol; *g*, plasmalogen-PtdCho. (*Inset*), % (relative to control) cellular PtdCho levels of PC-3 cells following treatment with Orlistat for the times indicated. B, representative ¹³C MR spectra of lipid extracts following 48 h of Orlistat treatment; peaks at 63–64 ppm have not been unequivocally identified. (*Inset*), % (relative to control) *de novo* PtdCho synthesis in PC-3 cells following Orlistat treatment for the indicated times.

Figure 5.

FASN inhibition is associated with a drop in ChoK activity (A) but not in long term inhibition of PI3K signaling (B). A. ^1H MR spectrum of cell lysates showing endogenous PCho and added Cho. *Inset*: representative plot of PCho synthesized by ChoK extracted from control cells and 48 h Orlistat-treated cells. Lines represent the straight-line fits to the data used to obtain rates of PCho synthesis ($R^2 = 0.89$, Control; $R^2 = 0.96$, Orlistat) indicating inhibition of ChoK following Orlistat treatment. B. Western blot analysis of P-Akt, Akt and GAPDH (loading control) expression in control and treated PC-3 cells following 3, 24 and 48 hours of Orlistat treatment.

Table 1

Summary of common affects of FASN inhibition on PC-3, MCF-7 and SKOV-3 cancer cells, expressed as % of control

Metabolite	Cells and treatment			
	PC-3 +orlistat ^a	SKOV-3 +orlistat ^b	MCF-7 +orlistat ^b	MCF-7 +cerulenin ^b
FA (<i>de novo</i>)	26±12 [*]	63±7	54±9	60±6
PCho (<i>de novo</i>)	41±23 [*]	69±21	37±12	79±12
PCho (total)	40±21 [*]	44±14	62±6	68±14
Choline (total)	53±32	82±33	51±15	52±4

* $P < 0.05$ (two-tailed unpaired Student's *t* test)

^a 3 or more repeats

^b 2 repeats.